A STUDY OF RNA SYNTHESIS AND ITS CONTROL IN ISOLATED MACRONUCLEI FROM TETRAHYMENA

A Thesis submitted to the University of Warwick
in fulfilment of the requirements for the
Degree of Doctor of Philosophy

by

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To

MY PARENTS

whose ideas have inspired me throughout,

and to

SALEEM

whose constant encouragement helped to produce this thesis.

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SUMMARY

1. Using the initiation inhibitors rifamycin AF/013 and heparin, it has been shown that isolated macronuclei from Tetrahymena are not capable of reinitiating RNA synthesis in vitro in the absence of cytoplasm. However, they do appear to be able to elongate RNA chains that were initiated in vivo.

2. Macronuclei were found to be able to synthesize discrete species of RNA under standard assay conditions. These RNA species have been characterised so far only in terms of their sizes. The species of RNA which have been observed on gel electrophoresis are 25S, 17S, 15-8S and 4-5S, together with one or two additional minor species. The 15-8S broad band, tentatively identified as mRNA, was only observed as a discrete peak in experiments in which nuclei were incubated in the presence of heparin. The other peaks have S values similar to ribosomal RNAs (25S, 17S and 5S) and transfer RNA (4S) as characterised in in vivo experiments.

3. It has been shown that Tetrahymena contains separate pools of free and template engaged RNA polymerase I and II. The size of these pools depends on the physiological state of the cells (growth conditions). Macronuclei from starved cells had 17% of the transcriptional activity of those from exponential cells, but it was shown that the former nuclei had a greater ratio of free to engaged RNA polymerase than the latter.

4. RNA synthesis in isolated macronuclei from exponential cells was stimulated on addition of cytoplasmic extract from rapidly dividing Tetrahymena cells. The stimulation was not very great and amounted to only 35% above control. However, RNA synthesis in macronuclei isolated from starved Tetrahymena cells was stimulated 100% by cytoplasmic extract from exponential cells.

It was also shown that RNA synthesis in isolated macronuclei could be stimulated by the cytoplasmic extracts obtained from Xenopus oocytes and chick embryo. The extent of stimulation in this case was about 80%.

It was concluded that at least some of the factors stimulating nuclear transcription were not species specific.

ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ATP	Adenosine 5'-triphosphate
GTP	Guanosine 5'-triphosphate
CTP	Cytidine 5'-triphosphate
UTP	Uridine 5'-triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
PPO	2,5-diphenyloxazole
POPOP	1,4- bis -(5-phenyloxazol-2-yl) benzene
SDS	Sodium dodecyl sulphate
RNase	Ribonuclease
Cyclic AMP	Cyclic adenosine 3':5'-monophosphate
Cyclic GMP	Cyclic guanosine 3':5'-monophosphate
DEAE	Diethyl amino ethyl
Tris-HCl	Tris(hydroxymethyl) amino methane hydrochloride
[M.E.S]	2 N-morpholino- ethane sulphuric acid
Poly [d(A-T)]	Polydeoxyadenylate-deoxythymidylate
rRNA	ribosomal RNA
mRNA	messenger RNA
tRNA	transfer RNA
S	Svedberg unit
g	Gravitational unit
cpm	Counts per minute
MW	Molecular weight
TCA	Trichloroacetic acid
Ci	Curie (3.7×10^{10} disintegrations per second)
poly(A)	polyadenylic acid
EGTA	Ethylene glycol bis (β-aminoethyl ether)-N-N,N'-tetracetic acid
NCS	Nuclear Chicago solubilizer

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

The process of transcription involves DNA-directed RNA synthesis which yields RNA (ribonucleic acids) with a base sequence complementary to that of the DNA (deoxyribonucleic acid) template. In this synthesis, RNA polymerase catalyzes the formation of inter-nucleotide 3' to 5' phosphodiester bonds, and thus plays a vital role in the transfer of information from DNA to RNA. Certain DNA sequences or genes when transcribed produce transfer RNA (tRNA) and others ribosomal RNA (rRNA), while some special DNA sequences code for the biosynthesis of messenger RNA (mRNA). Whilst the mRNA might be considered the means whereby genetic information is actually transmitted from the genome (the DNA), the rRNA and tRNA are essential for the working of the translational machinery. In contrast to bacterial systems, transcription and translation in eukaryotic cells are independent processes. In eukaryotes, the pre-mRNA synthesised in the nucleus is first processed and then passes from the nucleus to the cytoplasm for translation into proteins by cytoplasmic ribosomes. The control of transcription in the nuclei of eukaryotic cells is essentially a major mechanism of cellular regulation, and isolated nuclei provide a good cell free system in which to investigate the regulation of transcription at the molecular level.

1.2 Enzymes Involved in RNA Synthesis

The enzyme DNA-dependent RNA polymerase (systematic name: nucleoside triphosphate:RNA nucleotidyl transferase (DNA-dependent); E.C.2.7.7.6) catalyses the polymerisation reaction which results in the synthesis of RNA. For this reaction to occur, the enzyme

requires a DNA template and the four ribonucleoside triphosphates as substrates. The enzyme catalysed reaction has an absolute requirement for a divalent cation, usually magnesium or manganese. The reaction can be summarised as follows:-

$$pppPu + npppX \xrightarrow{DNA + Mg^{++}} pppPu(pX)_n + nppi$$

(pppX = ribonucleoside triphosphate, X = adenosine, guanosine, cytosine or uridine; Pu = purine ribonucleoside, ppi = inorganic pyrophosphate). The RNA product $pppPu(pX)_n$ is formed in a virtually irreversible reaction since pyrophosphorolysis of RNA has not been demonstrated with this enzyme (Hurwitz and August, 1963; Maitra and Hurwitz, 1967). The RNA synthesised by this enzyme in vitro is complementary to the DNA template used, and thus once RNA synthesis has been started the enzyme should copy the template accurately. Basically the synthesis of a RNA molecule starts at the 5' end and proceeds by the subsequent addition of ribonucleotides to the free 3' hydroxyl end of the growing chain. Thus, as shown in Fig.1.1,newly synthesised RNA has a triphosphate group at the 5' end and a free hydroxyl group at its 3' end.

RNA synthesising activity was first reported by Weiss (1960) in a nuclear system isolated from rat liver. In the early 1960's the existence of a DNA-dependent RNA synthesising enzyme was demonstrated in bacterial cells. Since then this enzyme activity has been demonstrated in many different types of cell (Hurwitz et al., 1960 ; Stevens, 1961). Much of the work on the mechanism and regulation of RNA synthesis has been done with the enzyme obtained from bacterial cells, and particularly with the purified RNA polymerase from E. coli (Burgess, 1969). It has been shown

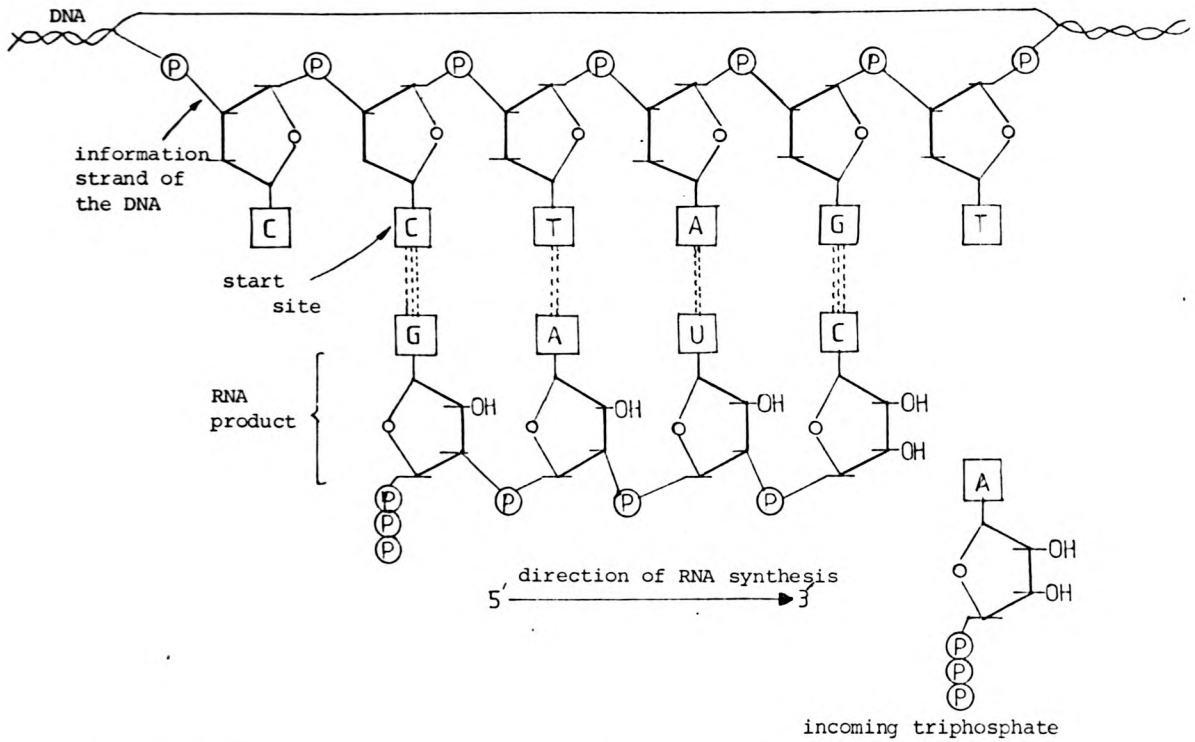


Fig. 1.1 A diagrammatic representation of the biosynthesis of RNA on one strand of DNA acting as template (Burdon, 1976)

that only one form of RNA polymerase is responsible for the synthesis of different types of RNA in prokaryotes. E. coli RNA polymerase has a molecular weight 480,000-500,000. It is made up of subunits and contains zinc. The subunit structure has been analysed by the technique of gel elctrophoresis in 6 M urea and 0.1% sodium dodecylsulphate. It is designated as β', β, σ, α and ω in order of decreasing molecular weight (Burgess et al., 1969). These subunits are present in the holoenzyme in the molar ratio, 2α (M.W. 39,000 each): 1β (M.W. 155,000): 1β' (M.W. 165,000): 1σ (M.W. 95,000), so the enzyme may be represented as $\alpha_2\beta\beta'\sigma$. The σ unit is an acidic polypeptide, required for the initiation of RNA synthesis at the "correct" promoter sties on the DNA template. This polypeptide can be easily dissociated from the holoenzyme aggregate to yield another form of enzyme, the core polymerase, as shown in Fig. 1.2.

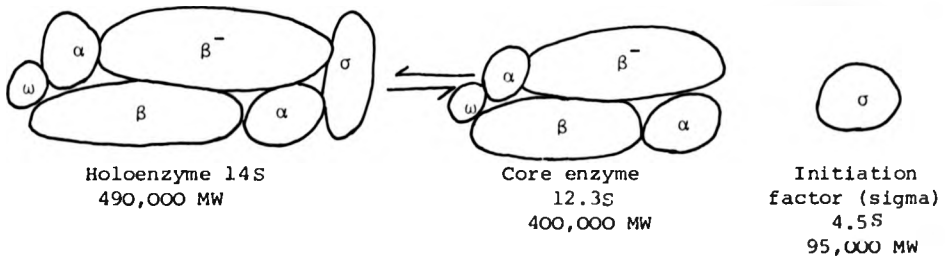


Fig.1.2 Summary of the subunit structure of E. coli: RNA polymerase (Burgess and Travers, 1970).

The β subunit is thought to be the target of the antibiotic rifampicin, suggesting its role in the initiation of RNA synthesis. The β' subunit is thought to be required for the binding of RNA polymerase to DNA (Heil and Zilling, 1970; Bautz, 1972). It has been shown that the 'core' enzyme will catalyze the synthesis of RNA chains from random sites on a DNA template in a non-specific way. Addition of σ factor to the core yields holoenzyme and restores selective initiation of RNA synthesis (Burgess, 1971; Bautz and Bautz, 1970). Once the σ polypeptide has recognised the correct promoter site on DNA, it is released from the complex of RNA polymerase and DNA, leaving the core enzyme to continue the synthesis of RNA. However, the termination at certain sites on the DNA template requires another protein, the rho (ρ) factor. This protein is a tetramer of polypeptide chains of molecular weight 50,000. The defined function of this factor has not yet been made clear, but it does allow the synthesis of RNA molecules *in vitro* of definite sizes, which are comparable to those produced *in vivo*. Thus this obligatory and temporal requirement of σ and ρ factors in transcription (Fig.1.3) may play an important role in the cellular control mechanism.

Following the discovery of multiple forms of RNA polymerases in rat liver nuclei by Roeder and Rutter (1969), a number of studies have been made which have revealed the presence of three different forms of RNA polymerases (I, II and III) in eukaryotic organisms and tissues. These include yeast (Adam *et al.*, 1972), the amphibian *Xenopus laevis* (Roeder, 1974), rat liver (Seifart *et al.*, 1972), calf thymus (Weil and Blatti, 1975) and human cells (Hossenlopp *et al.*, 1975). On the basis of this work, it is now well established that eukaryotic cells contain several distinct RNA polymerases which are localised in different sub-

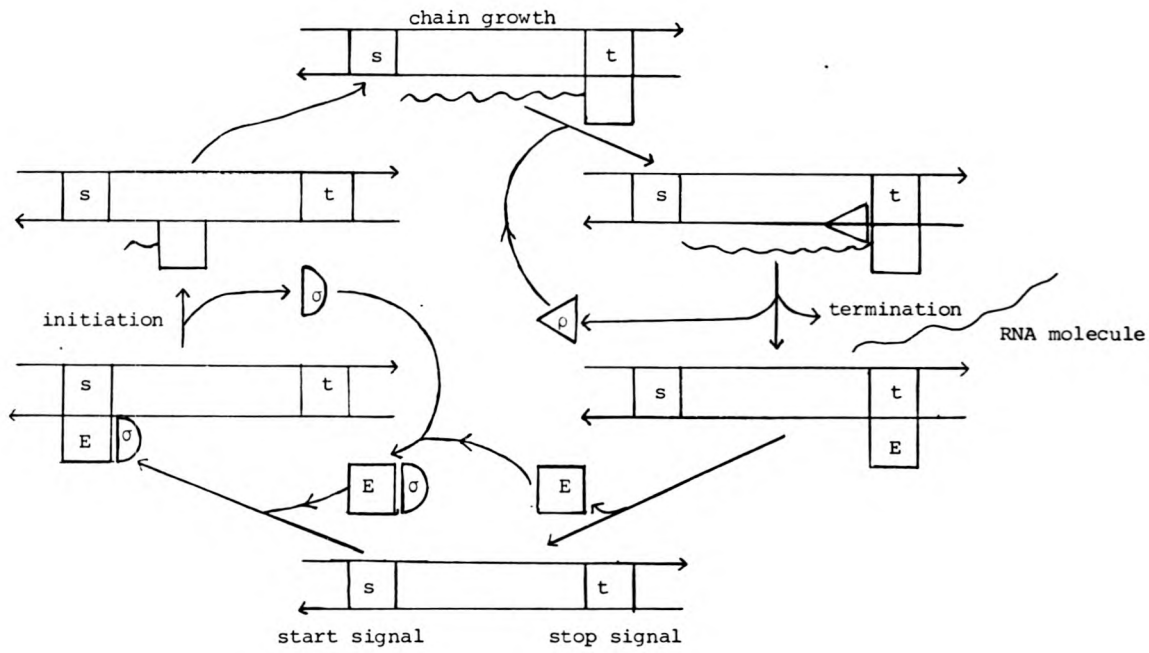


Fig. 1.3 Schematic representation for cyclical involvement of the transcriptional factors σ and ρ in the synthesis of a single RNA molecule

cellular fractions and play different functional roles in the cell. The species designated eukaryotic RNA polymerase I or A is normally found in nucleoli and accounts for ribosomal RNA synthesis, whereas RNA polymerase II or B and III or C are nucleoplasmic enzymes which are responsible for the synthesis of heterogeneous RNA (or pre-mRNA), 5S and transfer RNA respectively (Chambon, 1974). The differential sensitivity of the RNA polymerases to the mushroom toxin, α-amanitin, was first demonstrated by Stripe and Fume (1967) who showed that only nucleoplasmic RNA polymerase activity was totally inhibited by low concentrations of α-amanitin. This property has proved useful in analysing the different roles of eukaryotic RNA polymerases in transcription. Thus α-amanitin is now used as a specific inhibitor for eukaryotic RNA polymerase II just as rifampicin was used to inhibit the prokaryotic enzyme. The RNA polymerase II of the majority of eukaryotic organisms is sensitive at very low concentrations (10^{-9}-10^{-8} M), RNA polymerase III at higher concentrations (10^{-5}-10^{-4}), while RNA polymerase I is resistant to α-amanitin (Chambon, 1975). The analysis of the subunit structure of eukaryotic RNA polymerases has shown that they are multisubunit enzymes, each made up of two subunits of high molecular weight and several components of low molecular weight (less than 100,000). Sebastian et al.(1974) have suggested that some common structural features have been conserved during the evolution of these enzymes in prokaryotes and eukaryotes.

1.3 Mechanism of RNA Synthesis

It has been well documented in the case of the prokaryotic DNA-dependent-RNA polymerase reaction,that there are four identifiable steps involved in transcription when RNA polymerase

transcribes a DNA template. They occur in the following sequence:

1. Template site selection and activation: in which RNA polymerase binds to the DNA template, locates a specific site at which initiation can occur and assumes an active conformation.

2. RNA chain initiation: in which the enzyme catalyzes the coupling of ATP or GTP with a second ribonucleoside triphosphate and eliminates inorganic pyrophosphate to generate a dinucleoside tetraphosphate of the structure pppPupX; this moiety remains tightly bound to the RNA polymerase DNA complex.

3. RNA chain elongation: in which successive nucleoside-monophosphate residue are added from substrate nucleoside triphosphates to the initial dinucleoside tetraphosphate at its 3'-OH terminus in order to elongate the nascent RNA chain.

4. RNA chain termination and release: in which the nascent chain and the RNA polymerase are released from the template.

The overall reaction in vivo consists of complete synthetic cycle as shown in Fig. 1.4 on which a variety of control factors operate. In an in vitro system, the amount of RNA synthesised will depend on how much of this synthetic machinery is present (Chamberlin, 1976).

So far the majority of studies on the mechanism of transcription have been made in prokaryotic systems. In eukaryotes, the precise mechanism of RNA synthesis has not been so clearly established. Before one can hope to understand the regulation of RNA synthesis in detail, more studies will be needed to clarify the nature of the individual steps in transcription and the processing of RNA. The process of promoter selection is a key point at which cellular regulation takes place and the initiation of the RNA chains at a definite

THE TRANSCRIPTION CYCLE

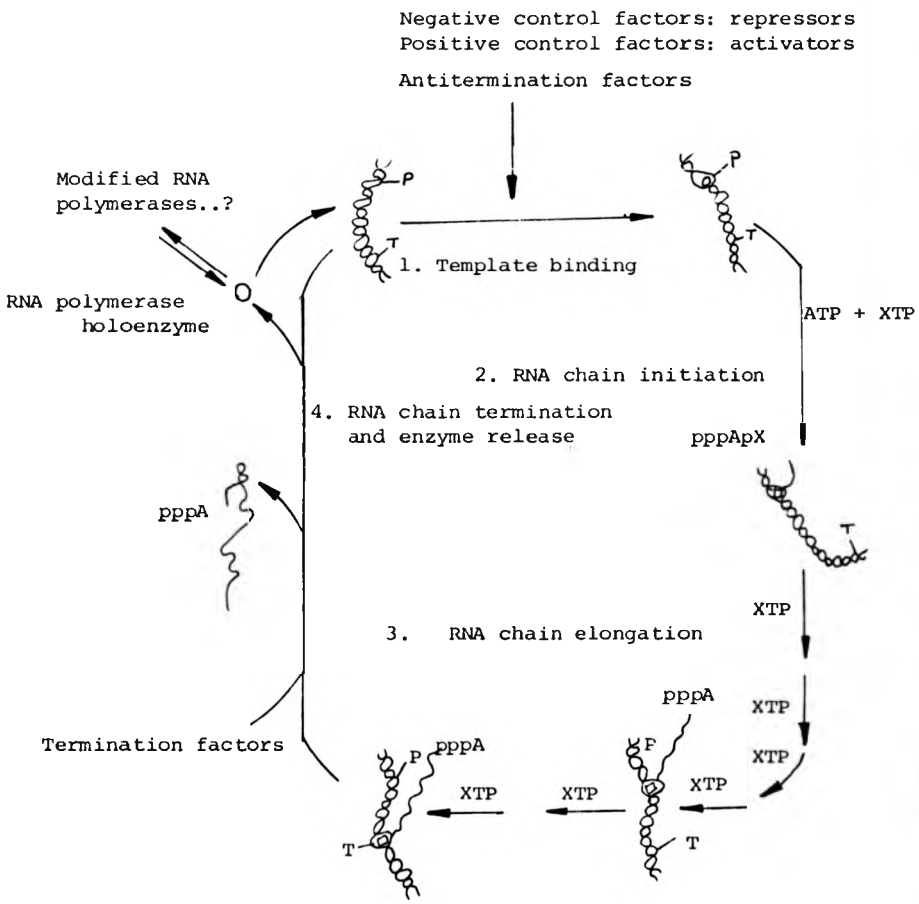


Figure 1.4 Diagrammatic representation of the individual steps involved in the synthesis of a single, specific RNA transcript by bacterial RNA polymerase (Chamberlin, 1976).

promoter site in the process of transcription. Thus the first two steps of the reaction sequence are of primary interest and will be discussed further.

Because of the general affinity of RNA polymerase for DNA, it can bind reversibly to DNA at specific sites, even in the absence of ribonucleoside triphosphates, to give a binary complex. Evidence has been presented for the existence of two states of these binary complexes when T7 DNA is transcribed by E. coli RNA polymerase (Mangel and Chamberlin, 1974). According to their model, complex I involves RNA polymerase binding at the promoter region, but the DNA in this region remains in a helical conformation. When complex II (RS) is formed, a limited strand separation (six to seven base pairs) occurs in this region of the DNA helix which allows the enzyme access to the base pairing residues of the DNA template. However, the transition from one state to the other occurs without dissociation of enzyme from the DNA. Several types of binding studies involving different techniques have demonstrated that binding of enzyme to template is dependent on ionic strength, pH and temperature, but not on magnesium ion concentration (Dubert and Hirschbein, 1969). It was shown that σ factor generally reduces the amount of non-specific binding of enzyme to DNA so that the RNA polymerase holoenzyme can bind efficiently only at the promoter site. Apparently tight binding of core polymerase in the absence of σ factor stabilizes the ternary enzyme-DNA-RNA complex during elongation. A 20-40 nucleotide stretch in DNA is required for the binding of an RNA polymerase molecule (Chamberlin, 1974). The initiation of RNA synthesis is quite distinct from the polymerization reaction, and involves the formation of the first internucleotide bond between two nucleoside triphosphates.

$$pppX + pppY \longrightarrow pppXpY + ppi \quad \text{(dinucleoside tetraphosphate)}$$

This dinucleotide is then elongated by subsequent addition of nucleoside monophosphate to the 3'-OH terminus in the chain elongation phase of the reaction:

$$pppXpY \xrightarrow{nZTP} ppXpY(pZ)n$$

However, the nature of the acceptor which bears the 3'-OH terminus in the case of initiation is a nucleoside triphosphate, whereas in the case of chain elongation this is the 3'-OH terminus of the growing chain (Chamberlin, 1974). The initiation of RNA synthesis requires local unwinding of melting of DNA before initiation and elongation can proceed (Saucier and Wang, 1972). A model was speculated as in Fig. 1.5.

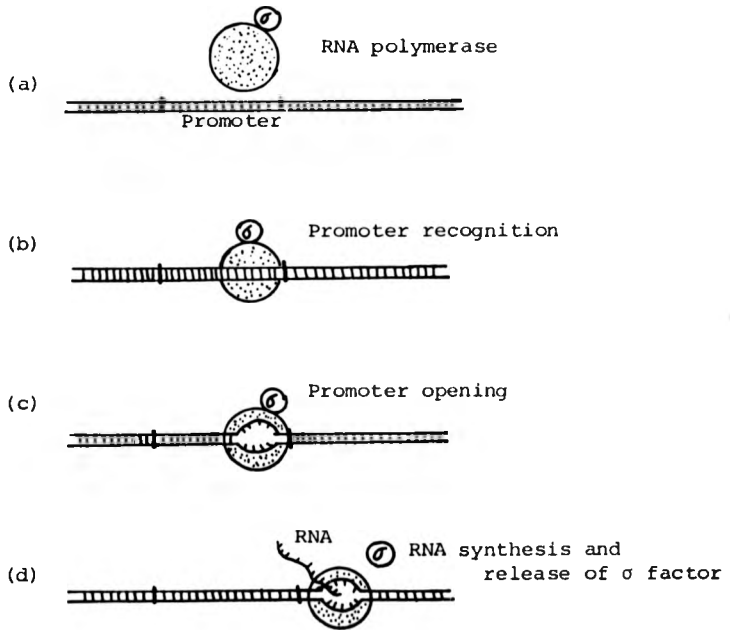


Figure 1.5 A diagrammatic representation of the stages in initiation of RNA synthesis

Furthermore, the bimolecular nature of the initiation reaction suggests that bacterial RNA polymerase must contain at least two kinds of binding sites for ribonucleoside triphosphates. One type of site binds specifically to the nucleoside triphosphate destined to become the 5' terminal end (initiation nucleotide site). The other site,which binds the nucleoside triphosphate to be added to the first, is designated the elongation nucleotide site. Krakow and Frank (1969) also showed, from studies of pyrophosphate exchange into β-γ position of nucleoside triphosphate, that the initiation binding site showed a greater specificity for ATP or GTP than the elongation site. RNA chain initiation has been studied by measuring the incorporation of γ-^{32}P labelled ATP or GTP into acid precipitable RNA, which retains the radiolabelled γ-phosphate at the 5' end (Maitra and Hurwitz, 1965). Moreover, kinetic studies on the effect of substrate concentration on RNA synthesis showed that the initiation of RNA synthesis required greater nucleotide concentrations than that required for elongation (Anthony et al., 1969). Kinetic studies have also given information about the role of σ factor in the initiation process in E. coli. Goff and Minkley(1970) showed that σ factor enhanced the specificity for strand selection as well as the number of RNA chains initiated. The assay technique for the measurement of chain initiation has recently been improved by the use of β-^{32}P GTP. Vennström et al. (1978) have studied the initiation of transcription in nuclei isolated from adenovirus infected cells using this nucleotide. Specific labelling of the 5' termini of RNA by β-^{32}P GTP occurred during the reaction and this experiment proved that the initiation of virus specific transcription could occur in isolated nuclei. Recently purine nucleoside 5'[γ-S] triphosphate has been used as an affinity probe

for studying the initiation of RNA synthesis *in vitro*. Reeve *et al*. (1977) showed that synthetic DNA templates were transcribed by *E. coli* RNA polymerase when nucleoside 5'[γ-S] triphosphate was used as one of the initiation nucleotide substrates. They showed that labelled sulphur was exclusively incorporated at the 5' terminus of RNA chain without affecting the rate of RNA sythesis. The transcripts initiated during this *in vitro* experiment could be rapidly isolated by their ability to bind covalently to a mercury agarose column. Other simple methods have been used to separate their initiation phase of transcription from elongation, and these will be discussed now in some detail.

The formation of the first phosphodiester bond in the chain initiation step converts the binary RNA polymerase-DNA complex into a ternary complex containing the nascent RNA chain elongated during the chain elongation phase of the synthetic reaction. It was demonstrated that high salt and low temperature reduced the stability of the binary complex; however, the ternary complex did not dissociate appreciably during chain elongation, even at high ionic strength (Richardson, 1966; Downey *et al*., 1970). Thus the chain elongation reaction involves elongation of nascent RNA chains by a single RNA polymerase molecule 'locked' into the ternary complex for the duration of chain elongation, until termination occurs. These ideas have been used to design assays for initiation which involve studies of transcription in the presence of different salt concentrations. Recently, it has become possible to measure some of the kinetic parameters of RNA chain initiation by employing specific inhibitors of this process. The most commonly used inhibitors are rifampicin and heparin, and both these compounds inhibit initiation but not elongation (Sippel and Hartmann, 1968).

Rifampicin is known to be a specific inhibitor of bacterial RNA polymerase while semisynthetic derivatives of rifamycin SV are efficient inhibitors of eukaryotic RNA polymerases. The most potent inhibitors have hydrophobic side chains, either long aliphatic side chains (AF/013 or AF/021) or benzyl groups (AF/05). However, the macrocyclic ring structure of rifamycin is apparently essential for inhibition because the side chains alone are inactive (Fig. 1.6). Meilhac et al. (1972) have studied in detail the mechanism of inhibition of eukaryotic RNA polymerases by rifamycin derivatives. They interpreted their results, using a model for the pre-initiation steps developed for E. coli RNA polymerases which can be represented as follows:

(a) Enz + DNA $\rightleftharpoons$ Enz. DNA (primary binding) Complex I

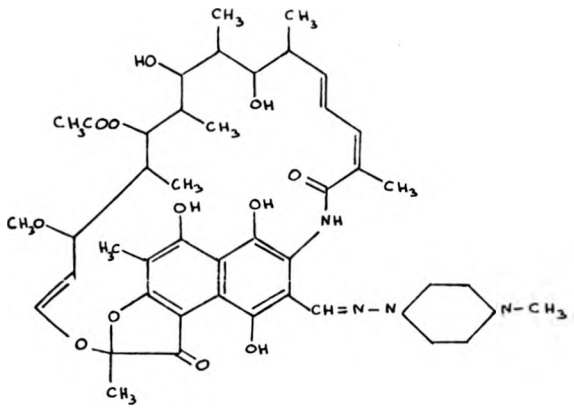
(b) Enz. DNA or Enz + DNA $\rightleftharpoons$ [Enz. DNA] (specific initiation site) Complex II

(c) [Enz. DNA] + σ $\underset{10^{\circ}C}{\overset{37^{\circ}C}{\rightleftharpoons}}$ Enz.σ.DNA (highly stable complex) Complex III

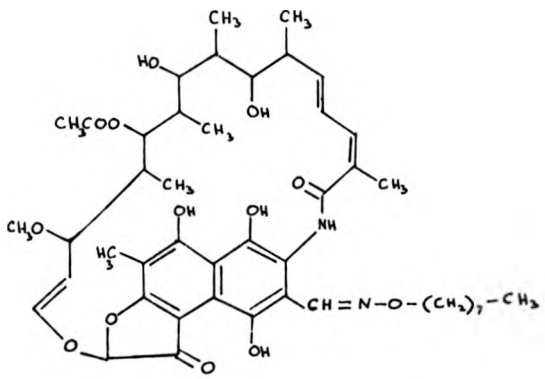
(d) Enz.σ.DNA $\rightleftharpoons$ [Enz.σ.DNA] (rifampicin resistant) Complex IV

(e) [Enz.σ.DNA] + pppPu $\rightleftharpoons$ Enz.σ.DNA.pppPu (initiation complex) Complex V

Scot and Downey (1970) have shown that when [d(A-T)] copolymer was transcribed by E. coli RNA polymerase, the initiation step involving the formation of the first phosphodiester bond resulted in the appearance of a stable enzyme-DNA complex. This complex showed resistance to rifampicin attack and did not dissociate in high salt. However, the earlier pre-initiation steps ((a) and (b)) were found to be very sensitive to rifampicin. Further more, the interaction of E. coli RNA polymerase with chick oviduct DNA and chromatin has been studied. It was shown that on preincubation of enzyme with chick DNA, stable pre-initiation complexes were formed which showed resistance to rifampicin in the presence of ribo-



Rifampicin



Rifamycin AF/013

Figure 1.6 Structure of some rifamycin - SV derivatives

nucleoside triphosphates. However, under these conditions RNA polymerase,non-specifically bound to DNA or free in solution,could not initiate RNA synthesis (Tsai et al., 1975). Rifamycin AF/013 has been used as a specific inhibitor of initiation in many different eukaryotic systems which include isolated chromatin from rat ventral prostate gland (Thomas et al., 1977) and isolated nucleoli and nuclei from Physarum polycephalum (Davies and Walker, 1978).

Divalent cations are essential for RNA synthesis. A differential effect of manganese and magnesium ions was observed for the initiation and elongation steps of the RNA polymerase I reaction. Using rifamycin AF/013, it was shown that Mn^{2+} but not Mg^{2+} was an effective initiation cofactor, while the elongation step required either Mg^{2+} or Mn^{2+} ions, but did not show any absolute specificity (Nagamine et al., 1978).

Heparin has been found to be an effective initiation inhibitor. It inactivates free RNA polymerases, but has no effect on the enzyme involved in RNA synthesis,which is tightly bound to a template. Heparin also appears to prevent the non-specific binding of RNA polymerase molecules to DNA (Walter et al., 1967). Inhibition by heparin depends upon the amount of enzyme present, and is independent of DNA template. It has been observed that 2 to 3 molecules of heparin bind to each molecule of RNA polymerase, suggesting that this binding either alters the conformation of the enzyme or blocks the sites normally used for DNA binding. Although the inhibition of initiation of heparin can be demonstrated in both prokaryotes and eukaryotes, the effect is more complex in the case of eukaryotes when isolated chromatin or nuclei are used. For example, a stimulatory effect of heparin on RNA synthesis has been observed in isolated rat liver nucleoli (Ferencz and Seifart,

1975). The stimulatory effect of heparin has been explained by Taylor and Cook (1977) who showed that it removes some histones protein from the condensed chromatin. This decondensation of chromatin activates the RNA synthesis on the template. Rifamycin AF/013 and heparin have been used as inhibitors of initiation in the work described here (Chapter 3).

1.4 Products of RNA Synthesis

(a) General patterns of RNA synthesis in the cell

In bacteria all three types of RNA, i.e. ribosomal (rRNA), transfer (tRNA) and messenger (mRNA) are synthesized by a single DNA-dependent RNA polymerase. In eukaryotes, however, there are at least three different forms of nuclear RNA polymerases, as well as a mitochondrial RNA polymerase, which all have specific localizations and functions in the cell. Most of the cellular RNA is synthesized in the nucleus and constitutes the primary transcription products, which are not identical to the functionally mature forms of RNA. These RNA species require extensive enzymatic processing or modification after transcription before the functional finished products, mRNAs, rRNAs and tRNAs are produced. The processes involve the following modifications:-

(a) nucleolytic reactions, such as cleavages and trimming of large precursor molecules; this process also involves the splicing of RNA-RNA ligation of mRNA molecules that has been discovered recently;

(b) terminal additions of nucleotides, such as occurs in 3'-poly-adenylation of mRNA, addition of CCA to the terminus of tRNA and 5'-capping of mRNA;

(c) nucleoside modifications, such as base or ribose methylation and the conversion of uridine to pseudouridine (Perry, 1976).

(i) Ribosomal RNA

Most bacteria possess roughly ten copies of the genes for ribosomal RNA, whereas animal cells possess DNA complementary to several hundred copies of ribosomal RNA (e.g. 260 in Drosophila, 900 in Xenopus, 1100 in HeLa cells (Maden, 1971). In bacteria, ribosomal RNA is synthesized as a single 30S pre-rRNA transcript of M.W. 2.1×10^6 (Nikolaev et al., 1974). The precursor molecule is processed to intermediate sized molecules, 17S and 25S, which then give rise to mature rRNA molecules of 16S and 23S (M.W. of 0.55×10^6 and 1.1×10^6 daltons respectively). In eukaryotes, the rDNA is located in the nucleolus, which is very active in the synthesis of rRNA. Scherrer and Darnell (1962) have demonstrated the presence of precursor rRNA with a sedimentation coefficient of 45S in HeLa cells. This precursor species was found to be very unstable and could be converted to mature forms of rRNA via several intermediates as shown in Fig. 1.7. (See next page).

While the 18S component (M.W. 0.7×10^6) is common to all animals, the larger 28S component has changed slightly with each major step of animal evolution. The larger component has a M.W. of 1.4×10^6 and 1.75×10^6 in sea urchins and mammals respectively (Loening, 1968). In prokaryotes and eukaryotes, a small rRNA with a sedimentation coefficient of 5S has been reported, which is transcribed from genes other than that major complementary genes for ribosomal RNA. A precursor molecule for 5S RNA has been found in E. coli cells (Forget and Jorda, 1970).

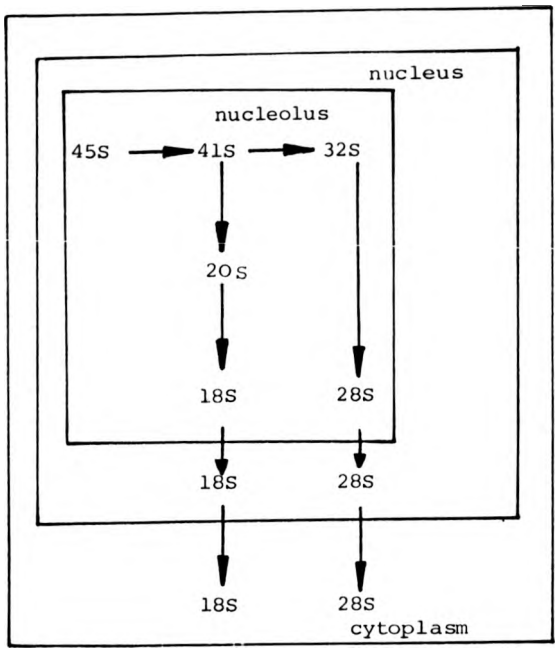


Fig. 1.7 A schematic diagram of the intracellular location of the basic steps believed to be involved in the post transcriptional processing of 45S ribosomal precursors in HeLa cells.

(ii) Messenger RNA

Messenger RNA is transcribed from one of the strands of the DNA helix in prokaryotes. Pulse labelling studies in E. coli have shown that the earliest observable precursors of mRNA are heterogeneous with sizes between 8S and 30S (Blundell and Kennell, 1974). mRNA is metabolically unstable in E. coli and has a half-life of one to two min. In eukaryotes, heterogeneous nuclear RNA is

synthesized in the nucleoplasmic fraction and can be identified by the rapid incorporation of radioactive RNA precursors by the polyadenylated 3' terminus, and by the polydisperse size distribution (10-100S). This hnRNA has a high turnover rate (half-life of about 20-60 min) and can be distinguished from the rRNA precursor by its low G : C ratio.

It has been demonstrated in mammalian cells that hnRNA is the precursor of cytoplasmic mRNA (500-5000 nucleotides long) (Jalinek et al., 1973). Messenger RNAs of eukaryotic cells can be distinguished by the poly(A) sequence (60-200 nucleotides long) attached to the 3'-OH end. The one known exception is the histone mRNAs of higher eukaryotes which does not have any poly(A) tail (Brawerman, 1974). Poly(A) sequence apparently plays an important role in the processing or transport of mRNA. Some messenger RNA has also been shown to possess a N-7 methylguanosine structure as a cap at the 5' terminal end (Adam and Cory, 1975).

(iii) Transfer RNA (tRNA)

Synthesis of precursor tRNA has been shown in E. coli (Altam, 1971) and in eukaryotes (Burdon, 1971). These molecules are processed to mature tRNA. It was demonstrated that pre-tRNA had a molecular weight which was greater than normal transfer RNA and contained 30-39 extra nucleotides. Thus maturation of pre-tRNA involves (a) trimming of the molecule to tRNA dimensions and (b) modification to the primary structure as a result of nucleoside methylation. This is depicted in Fig. 1.8. (See next page).

In general, tRNA have molecular weights in the range of 0.23 x 10^5 to 0.28 x 10^5 and sedimentation coefficients of about 4S. The length of tRNA chains ranges from 76-85 nucleotides.

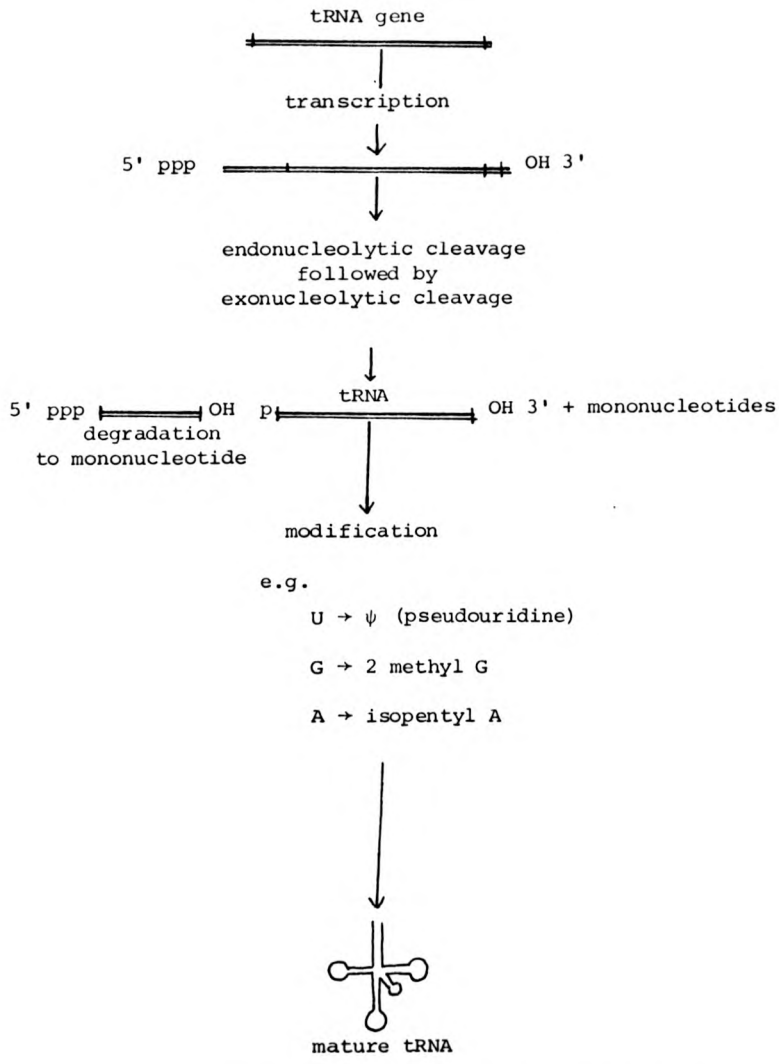


Fig. 1.8 Steps in the processing of a monocistronic pre-tRNA molecule into mature tRNA

(b) Studies on transcription products

In general, progress in understanding the processing of RNA and the control of RNA synthesis has been achieved by combining the information obtained from in vitro and in vivo experiments. Several studies have been made to characterize and compare the RNA synthesised in whole cells (in vivo) and in isolated nuclei and on isolated chromatin (in vitro).

Markyanka and Gould (1973) have shown that the in vitro product of transcription of rat liver chromatin with homologous rat liver RNA polymerase II was high molecular weight RNA with a sedimentation coefficient of 18-45S, which was also comparable with the product from isolated nuclei. However, they found much smaller products (10S RNA) when bacterial enzyme was used. Isolated myeloma nuclei and nucleoli were shown to be capable of RNA synthesis when incubated with nucleotides in an in vitro assay system. Weinmann and Roeder (1974) have detected pre-4S and 5S RNA species when they analyzed the newly synthesized nuclear RNA on the basis of their sedimentation coefficient values. Thus they were able to demonstrate the presence of RNA polymerase III in isolated nuclei which faithfully transcribes the tRNA and 5S RNA genes.

The RNA synthesized by RNA polymerase I in isolated mouse cell nuclei was found to be a precursor of ribosomal RNA and had a sedimentation coefficient of 45S (Harmon and Gurney, 1976). A heterogeneous population of RNA species (9-13S) was also synthesized by RNA polymerase II in this system. Recently Mory and Gefter (1977) have analyzed the transcription products synthesized in isolated nuclei from mouse myeloma cells under appropriate conditions. They also showed the heterogeneous nature of the RNA synthesized, which ranged from smaller than 10S to

larger than 45S. This range of RNA products resembled those made *in vivo* in size at least.

Studies of this type have also been extended to unicellular eukaryotes. At present, the majority of the work reported is on the synthesis and processing of rRNA, and comparison can be made with these processes in eukaryotes. A detailed *in vivo* study has shown that precursor ribosomal RNA synthesized in the yeast nucleus, *Sacchromyces cerevisiae*, has a sedimentation coefficient of 37S. This precursor is subsequently processed into 17S and 26S rRNA species in the cytoplasm (Sillevis Smith *et al.*, 1972). A similar kind of study has been made with *Tetrahymena pyriformis* by Kumar in 1970, who showed by means of pulse labelling with [^{3}H]-labelled uridine that a 35S rRNA precursor was synthesized in the nucleus. This precursor was processed and appeared in the cytoplasm as 26S and 17S RNA. Recently such r-RNA precursor molecules have been isolated from both starved and refed cells of *Tetrahymena pyriformis*. It was confirmed that 35S rRNA was the primary transcript of rRNA genes, and that 25S and 17S mature rRNA species were derived from this precursor (Niles, 1978).

From these results it appears that rRNA synthesis and processing is much the same in higher and lower eukaryotes, except that there might be a small difference in the size of the precursor molecule and processed intermediates associated with each particular organism.

1.5 Transcriptional controls in prokaryotes

The transcriptional unit in bacteria is called an operon, and its component structural genes are transcribed into a single polygenic messenger RNA. The concept of the operon and its involvement in the control of transcription in prokaryotic systems was first

introduced by Jacob and Monod in 1961. According to this model, operons are under negative feed back control, as they are specifically inhibited by repressors of the regulatory genes. Such repressors have been isolated in the case of the lac operon (Gilbert and Müler-Hill, 1967). Besides lac, other operons which are found to be under negative controls are the gal, arg, try and his operons. The region on the DNA which controls RNA polymerase binding and RNA chain initiation is called the promoter. The structural gene has an initiation point known as the operator, which in combination with repressor blocks the transcription of the whole operon. Both the binding of RNA polymerase to the promoter region and the attachment of repressor to its unique operator site involve specific regulation of a particular DNA sequence by proteins.

The binding of various allosteric effectors,known as inducers, to these repressors produces conformational changes which result in a loss of repressor affinity for DNA. Thus the transcriptional activity of an inactivated operon can be restored since the binding of a repressor is reversible. Positive control of the lac operon is exerted through the phenomenon termed catabolite repression. Studies on the DNA-directed cell free synthesis of enzymes of the lac operon showed that cyclic AMP has a stimulatory effect on the expression of the lac operon. This effect can be explained on the basis of the model proposed by Zubay et al. in 1970. According to this model, cAMP is required to activate the gene activating protein (CAP) and stimulates its binding to RNA polymerase, probably in the promoter region. This complex is then active in the initiation of transcription of the operon, such as lac operon as depicted in the figure on the next page (Figure 1.9).

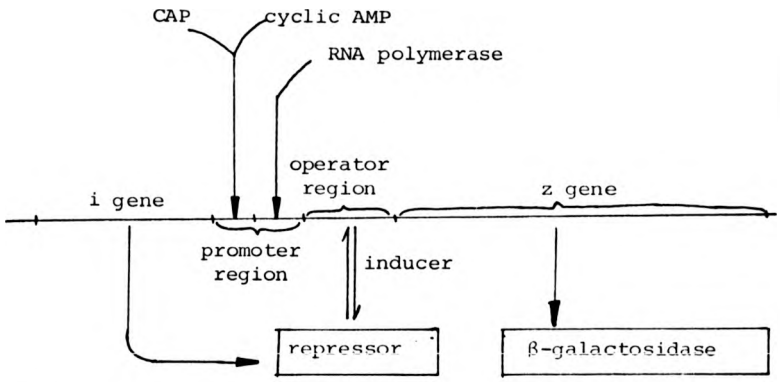


Figure 1.9 The lac region of the E. coli chromosome

Several protein factors have been isolated from E. coli which modulate the transcription in vitro (Voigt, 1969). These protein factors have effects on initiation, elongation and termination steps during RNA synthesis. Burgess et al.,in 1969, have isolated a protein factor, sigma (σ) from E. coli. This factor is involved in stimulating the initiation of RNA synthesis in vivo and in vitro, and has been well characterized. Another factor called rho (ρ) has been isolated from E. coli, which terminates newly synthesised RNA chains (Roberts, 1969). It was shown that ρ factor causes termination at DNA sites which are not recognised by the polymerase alone. Some ideas on the mechanism of sigma (σ) and rho (ρ) factors in the regulation of RNA synthesis have already been described in Section 1.1. Some other protein factors isolated are M factor, which stimulates the frequency of initiation (Davidson et al., 1969), psi (ψ) factor which specifically initiates ribosomal RNA synthesis (Travers et al., 1970) and K factor which terminates RNA chains at sites different from those where ρ factor acts (Schafer and Zilling, 1973). There is evidence for the existence of other types of control mechanism; for example, the synthesis of ribosomal RNA in bacteria is under

stringent control. This control is exerted in response to different nutritional conditions. For example, amino acid starvation of certain strains of bacteria causes a decrease in rRNA synthesis. This decrease is due to the rapid intracellular accumulation of ppGpp (guanosine tetraphosphate) (Cashel, 1969), since the concentration of this nucleoside appears to be inversely correlated with rRNA synthesis. Although the mechanism of these effector substances is not clear, they may alter the properties of RNA polymerase itself as suggested by Travers (1970).

1.6 Transcriptional controls in eukaryotes

(a) Eukaryotic chromatin

The transcriptional unit in eukaryotes is much more complex than in prokaryotes (Yunis and Yusmineh, 1971). The genomic DNA of virtually all eukaryotes is contained within a membrane bound nucleus and it is packaged there with proteins and a small amount of RNA to form a complex substance called chromatin. A model of the chromosome structure of higher organisms was proposed by Crick in 1971. According to this model, regions of compact nucleoprotein (globular DNA) function as the controlling elements, and loosely arranged nucleoprotein (fibrous DNA) represents the coding regions for RNA. A more recent model for the structure and function of the repeat units in chromatin was proposed by Kornberg in 1974, and is depicted in Figure 1.10.

More recently, in 1977 Kornberg studied the detailed structure of the repeat unit which is now termed a nucleosome. According to the suggested structure, a nucleosome is made up of eight histone molecules (two each of types F2A1, F3, F2A2 and F2B) complexed with 200 base pairs of DNA. In addition, a fifth histone, H1, is found to be associated with the nucleosome of most, but not all cell types. The DNA component of the nucleosome is made up of a 'core' of 140 base pairs, and is highly conserved in different species,

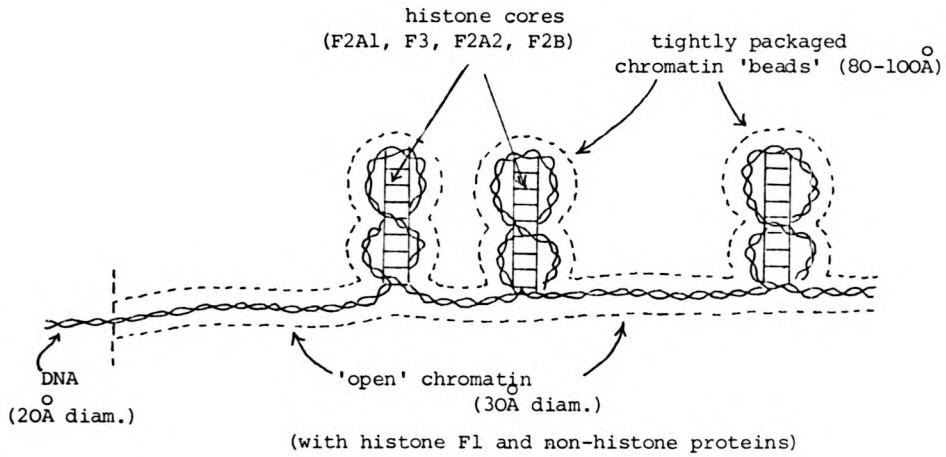


Fig. 1.10 A highly schematic diagram of mammalian chromatin structure

(Kornberg, 1974)

and a 'linker' which varies from 15 to 100 base pairs, depending on the cell type.

(b) The role of histones and non-histones proteins in transcription

There is a growing evidence that chromosomal proteins play an important role in determining the structural and functional properties of the eukaryotic genome (Elgin and Weintraub, 1975), and that the state of the DNA in chromatin and the nature of its interaction with histone proteins has important implications for transcriptional control (Stein et al., 1974). Histones are now considered to be non-specific repressors of transcription and act by binding to DNA and restricting the RNA synthesis. However, these proteins cannot be responsible for the fine control of RNA synthesis since there are only five types of histone and the arrangement of histones in chromatin in different cell types is usually identical. The inhibition of RNA synthesis by histones can be counteracted by non-histone proteins which have been postulated to have a role in derepression (Paul and Gilmour, 1968). Non-histone proteins are a heterogeneous class of molecules, associated with chromatin structure which are tissue and species specific (Platz et al., 1970). The interaction of different types of non-histone proteins with DNA is also tissue specific and can thereby bring about specific gene activation. Unlike histones, the cellular concentration of non-histone proteins reflects the changes in the metabolic activity of cells. Moreover, it has been demonstrated that these proteins are preferentially localized in the actively transcribing region of chromatin (Hill et al., 1971). The stimulation of transcription by non-histone proteins has also been demonstrated in a cell free system (Sperlsberg et al., 1971).

It has been demonstrated that chemical modifications of

histone and non-histone proteins alter the structure of chromatin and can thus regulate transcription. The role of histone phosphorylation and acetylation (Kleinsmith et al., 1966) as well as the phosphorylation of non-histone chromosomal proteins in the control of transcription in vivo and in vitro is now well established. Although histones can be phosphorylated to a small extent, the non-histone proteins are extensively phosphorylated and dephosphorylated during the cell cycle. In the case of histone gene transcription, the phosphorylation of a class of nuclear proteins has been postulated to control gene expression. A general mechanism has been suggested for gene activation in which the phosphorylation of specific nuclear proteins causes the derepression or the DNA template. This mechanism is depicted in the models in Figures (1.11) and (1.12), taken from the report of Kleinsmith (1975).

1.7 Transcriptional controls in eukaryotes at the RNA polymerase level

Three major ways in which direct controls on RNA polymerase could be exerted have been proposed:

(i) modulation of the total RNA polymerase synthesised

(ii) modulation of the specific activities of preformed RNA polymerase molecules (i.e. by interaction with effector substances

(iii) changes in the proportion of RNA polymerase bound to template and actively transcribing, compared with the soluble pool of enzyme not immediately involved in transcription (Beebee and Butterworth, 1977).

The latter two modes of control will be discussed in some detail since in the present study experiments have been designed to test their involvement in the control of RNA synthesis in Tetrahymena.

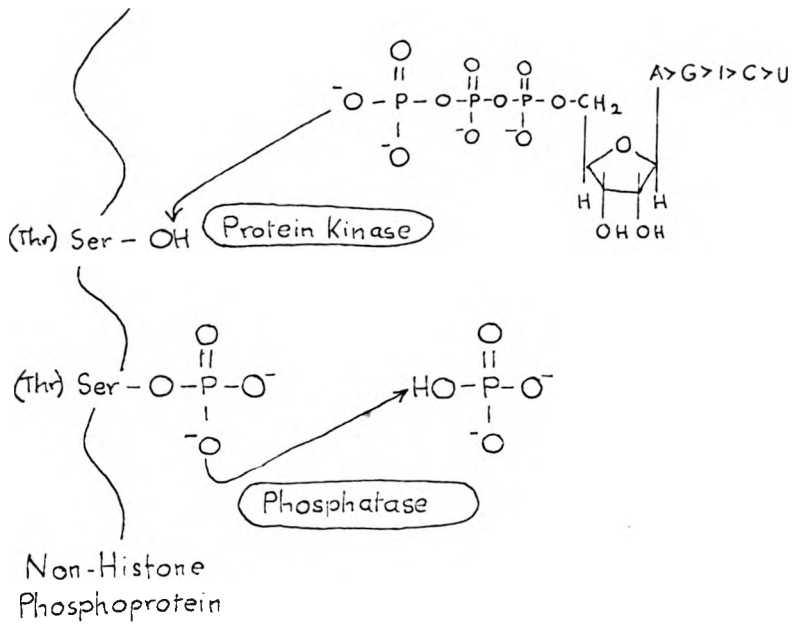


Fig. 1.11 Model summarising the relationships between the phosphorylation and dephosphorylation reactions of non-histone phosphoproteins (Kleinsmith, 1975)

In this model, serine (and threonine) residues in the proteins are phosphorylated via the terminal phosphate of various nucleoside triphosphates and deoxynucleoside triphosphates in a typical protein kinase reaction. In a separate phosphatase reaction, the phosphoserine (and phosphothreonine) bonds are broken, releasing inorganic phosphate.

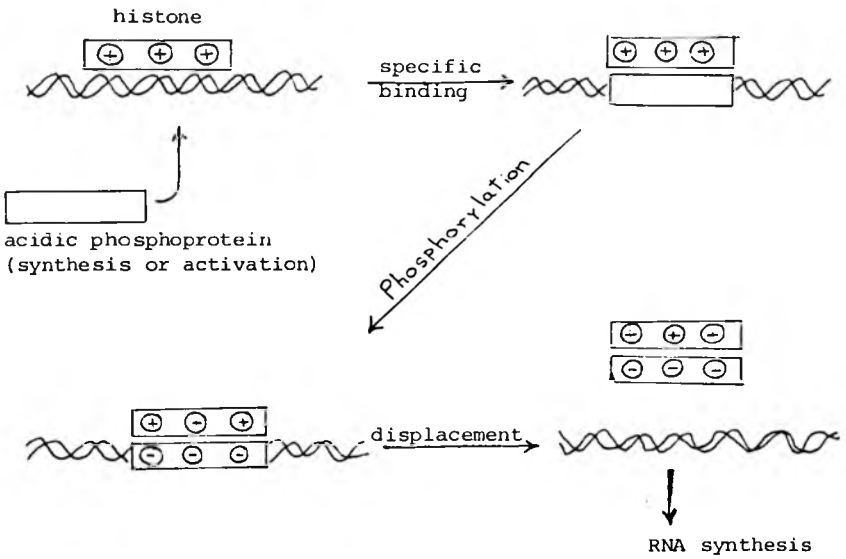


Fig. 1.12 A model shows some of the properties of non-histone phosphoproteins in gene activation. This model is based on the specific binding of non-histone proteins to DNA, their subsequent phosphorylation and finally histone displacement. The resulting naked DNA is then capable of synthesising RNA (Kleinsmith, 1975)

Briefly, this model is based on the specific binding of non-histone proteins to DNA and their subsequent phosphorylation. The negatively charged phosphate groups of the phosphoprotein might interact with the positively charged histones, thereby displacing the inhibitory histones from the DNA-histone complex and thus allowing the DNA to become active as a template for RNA synthesis.

(a) Free and engaged forms of RNA polymerase

The existence of pools of 'free' RNA polymerase which are inactive towards the endogenous template was demonstrated by Bagshaw and Malt in 1971. They showed that addition of denatured DNA to isolated rat nuclei stimulated the *in vitro* RNA polymerase activity. This observation opened the possibility that there might be some free enzyme which could become active on addition of exogenous template. Later, similar conclusions were drawn from the studies done by Yu and Feigelson (1971; 1972). Yu, in 1974, presented convincing evidence for the existence of two functional states of RNA polymerase. He demonstrated the presence of a transcriptionally active state of the enzyme (engaged enzyme) in isolated rat liver nuclei and nucleoli. Free RNA polymerase activity was measured on synthetic templates such as poly(dc) or poly[d(A-T)], under conditions where template bound RNA polymerase activity was blocked by actinomycin D.

A model for the mechanism of interaction of actinomycin D with DNA was proposed by Hamilton in 1963. Later, another model was proposed by Warning (1968). They postulated an explanation for the specificity of actinomycin in terms of its intercalating between deoxyguanosine residues of DNA. This interaction involves the minor groove of helical DNA as depicted in Figure 1.13. Synthetic templates are effective in directing RNA synthesis in the presence of actinomycin D, because they do not contain deoxyguanosine residues. Actinomycin D blocks pre-engaged enzyme from continuing the elongation of RNA chains already initiated *in vivo*. It does this by intercalating in the DNA duplex in deoxyguanosine rich sequences to an extent which eliminates any further movement of the transcription complex.

The existence of free RNA polymerase in nuclei or nucleoli may

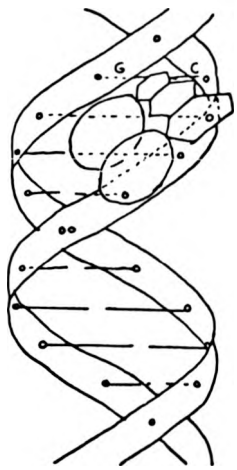
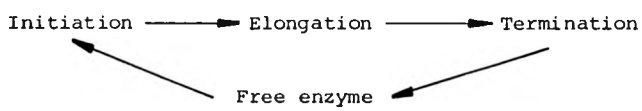


Fig. 1.13 Schematic representation of a model for the actinomycin-DNA complex taken from the reference (Warning, 1968)

Intercalating agent actinomycin D, when it binds to duplex DNA, the flat phenoxazone ring system intercalates between two successive guanine-cytosine pairs while two cyclic pentapeptide chains lie in the minor groove of a double helix, each hydrogen bonded to the opposite DNA chain.

represent a normal phase of an RNA polymerase cycle, at least under in vivo conditions where proper initiation and termination of RNA chains is occurring.



Changes in the pool size of free and engaged enzyme have been demonstrated in cells under conditions where major changes have occurred in the rate of RNA synthesis. The rate of the ribosomal RNA synthesis is greatly affected in different eukaryotic organisms by (1) treatment with cycloheximide (Lampert and Feigelson, 1974), (2) partial hepatetomy (Yu, 1975) and (3) amino acid starvation (Grummt, 1976). Variation in the pool size of free and engaged RNA polymerase may be a controlling factor during development (Davidson, 1968; Bramachary, 1973). This hypothesis was further supported by Colman in 1975, who studied the transcription in Xenopus laevis in the presence of exogenous poly[d(A-T)] microinjected into eggs and oocytes. A large stimulation of RNA synthesis was observed when unfertilized Xenopus eggs were injected with poly[d(A-T)], whereas occytes showed far less stimulation toward this exogenous template.

The oocyteshavea high rate of RNA synthesis compared to the mature unfertilized egg. There thus appear to be special factors which control the pool size of free and engaged enzyme in the oocyte relative to the egg.

(b) Putative factors modulating RNA polymerase activity

The discovery of sigma factor by Burgess et al. (1969) made it possible to begin to understand the control of transcription in prokaryotes. This is the first fine controlling factor found in living cells that can act specifically to turn on genes. So far, very little

is known about the role of controlling factors in transcription in eukaryotes. It was first reported by Mondal et al. (1972) that initiation and elongation factors from coconut chromatin were able to stimulate the homologous RNA polymerase activities in isolated nuclei. The presence of the pi factor in yeast cell was described by Dimauro et al. (1972). This factor was found to bring about the binding of RNA polymerase to certain DNA sequence. Thus it acts as an initiation factor for RNA synthesis. Many studies have involved the isolation of proteins from cytoplasm which have stimulatory effects on transcription by purified RNA polymerases. These stimulatory factors have been reported to occur in animal systems such as calf thymus (Stein and Hausen, 1970) and rat liver (Seifart et al., 1973). It was shown that these factors are rather specific for non-ribosomal (form II) RNA polymerase and appear to act at the elongation stage. Further studies have demonstrated the presence of such factors in mouse myeloma cells (Lentfer and Lezius, 1972), ascites cells (Lee and Dahmus, 1973) and HeLa and KB cells (Sughen and Keller, 1973). Most of the reports so far presented are based on the stimulatory effects of factors on transcription, using a system of purified enzyme and isolated DNA. However, isolated nuclei and chromatin have proved to be useful in vitro systems for studying the effect of these protein factors. Stimulation of RNA synthesis in isolated mouse liver nuclei in vitro was first reported with cytoplasm derived from mouse TLT cells (taper-liver tumour), L cells and from normal mouse liver cells. (Thompson and McCarthy, 1968). They showed that stimulation was greater in isolated nuclei when incubated with cytoplasm derived from the cells which were rapildy dividing. Further studies on the effect of cytoplasm on RNA synthesis in isolated nuclei showed that very long transcription times could be achieved after addition of

cytoplasm (Wu and Zubay, 1974; McNamara et al., 1975). In these earlier studies no attempt was made to fractionate the cytoplasm. Recently Maclean and Hilder in 1977 have also reported the stimulation of RNA synthesis in isolated nuclei from Xenopus erythrocytes, when incubated with cytoplasmic extracts from different sources. They further fractionated the cytoplasmic extracts from rat liver and demonstrated that the active factor was protein of molecular weight 40,000 Daltons.

To fully understand the role of any stimulatory factor, one needs to purify it and then determine (1) whether it is specific for transcription by RNA polymerase I, II or III, (2) whether it binds to RNA polymerase or template, (3) whether it effects either initiation, elongation or termination and (4) whether it is produced in the cell under environmental conditions which favour rapid RNA synthesis and whether it disappears from the cytoplasm under conditions where there is little RNA synthesis. As yet, few of these questions have been answered for any eukaryotic stimulatory factor. Thus the mechanism of control of transcription in eukaryotes under in vivo conditions has still to be worked out, and the function and importance of cytoplasmic factors which stimulate transcription in in vitro systems has yet to be established.

1.8 Tetrahymena pyrifomis

(a) Tetrahymena as a model system for studying transcription

Tetrahymena pyriformis is a unicellular eukaryotic organism. It has been used in biochemical and physiolgical studies of the cell cycle and studies of the control of cell growth and division (Hill, 1972; Elliot, 1973). Its advantages as a model system for such studies are (i) it has a short generation time (2½ hr to 3 hr under most conditions), (ii) it can be grown in large quantities, (iii) mass cultures of Tetrahymena pyriformis are amenable to

synchronization and (iv) studies can also be made easily on isolated subcellular systems.

(b) General background and morphology

Tetrahymena is an organism with a pyriform or pear-shaped ciliated cell. The average cell size is about 50 x 30 µm but size varies with environmental conditions. Most of the cell surface is covered with sixteen to twenty rows of cilia (primary meridians) which lie parallel to the long axis of the cell and alternate with secondary meridians of mucocyst pores. The extreme anterior and posterior ends are bare. The buccal or oral apparatus lies towards the anterior end and the anal pore lies on the posterior, ventral body surface. The macronucleus is typically oval to spherical body and always centrally positioned (Fig. 1.14). Tetrahymena has typical euaryotic organelles including a nucleus, mitochondria, endoplasmic reticulum and food vacuoles. The change in mitochondrial shape and distribution have been correlated with changes in cell age, and thus cellular activity. In early exponential growing cultures the mitochondria are minimal in number, oval shape and peripherally located near the basal bodies of the cilia, but become numerous, uniformly spherical and randomly distributed throughout the cytoplasm in the stationary phase culture. Such variation in the mitochondrial distribution may be co-ordinated with the function of these organelles.

Two basic types of endoplasmic reticulum have been described in this protozoan. One is the smooth (agranular) endoplasmic reticulum, devoid of ribosomes, but containing the Golgi apparatus. The other is the rough (granular) endoplasmic reticulum and contains ribosomes.

Microbodies called peroxisomes are also found in Tetrahymena which contain enzymes catalyzing the oxidation of


OA
PM
SM
1
OA
M
2

Fig. 1.14.1

A Chatton-Lwoff silver stain of a cell from a logarithmic culture. The oral area (OA) is in the anterior quarter of the cell. The dark granules of the primary meridians (PM) represent sites of cilia. Alternating with them are secondary meridians (SM) composed of mucocysts pores. x 480

Fig. 1.14.2

A longitudinal section through a stationary-phase cell (strain GL). The oral area (OA) is in the anterior of the cell. The macronucleus (MA) is ventrally located. Peripherally, the mucocysts (MU) and mitochondria (M) are indicated. x 1500

Reproduced from reference, Elliot (1973)


OA
PM
SM
1
OA
M
2

α-hydroxy and α-amino acids. Hydrogen peroxide formed by these enzymes is destroyed by catalase. Also present in these particulate organelles are the glyoxylate cycle enzymes, which play a role in the carbohydrate metabolism of this protozoan by converting lipids to glycogen. More than one type of lysosome are found to exist in Tetrahymena . These are primary lysosomes (food vacuoles) and tertiary ones, which contain other subcellular organelles (Elliot, 1973).

Like most of the ciliated protozoans, the vegetative cell of Tetrahymena pyriformis contains two distinct types of nuclei, a diploid micronucleus and a large polyploid macronucleus. In both micronucleate and amicronucleate strains a single oval macronucleus, about 10 μm in diameter, is located in the central portion of the cell. It contains more than one hundred times the amount of DNA present in a micronucleus, and the chromatin contains histones H1 and H3, which are absent from the micronucleus. In sexually active strains, there is a spherical micronucleus which is located near the macronucleus.

Since studies were carried out on the amicronucleate strain of Tetrahymena pyriformis W, the macronucleus will be described in further detail.

(i) Macronucleus

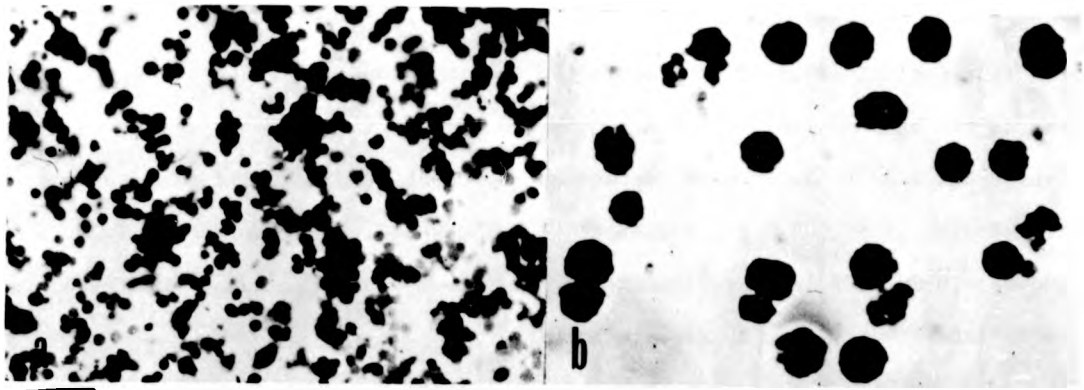
The following description is based on interpretations of electron micrographs of the macronucleus. This organelle is surrounded by a porous envelope consisting of two distinct unit membranes with a space between them. The overall thickness of the envelope is approximately 200 nm. The outer membrane of the envelope is contiguous with the endoplasmic reticulum, thus providing a continuous channel from the space in the nuclear envelope to the cisternae of the endoplasmic reticulum. The

nucleoplasm of the interphase macronucleus contains small granular, evenly distributed chromatin granules and larger peripheral nucleoli as shown in Fig.1.15, taken from the reference Higashinakagawa and Mita (1973). It is evident from electron microscopy and cytochemistry that these bodies contain the bulk of macronuclear DNA (Elliot, 1973). Structural changes occur in the chromatin bodies during the cell cycle and in response to environmental changes (Nilson, 1970). The nucleoli lie close to the inner membrane of the envelope; they consist of numerous dense granules resembling cytoplasmic ribosomes arranged around a larger, more dense region which has been identified as the site of nucleolar DNA. Furthermore, in Tetrahymena the nucleolar organisation has been observed to change under certain environmental growth condition by the phenomenon of aggregation-disaggregation of these fused granules. The change in appearance and structure of these granules during the cell cycle has also been observed (Satir and Dirkson, 1971).

The isolation of Tetrahymena macronuclei is relatively simple and several methods for their isolation have been reported (Lee and Scherbaum, 1965; Mita et al., 1966; Muramatsu, 1970; Everhart, 1972; Gorovsky, 1970). Isolated macronuclei provide a good in vitro system in which to study transcription and its controls.

(c) RNA polymerase and RNA synthesis

Grovsky and Woodward (1969) demonstrated by means of labelling and radioautographic studies that the macronucleus is the major site for the synthesis of cellular ribonucleic acid. Later, in 1970, Lee and Byfield first isolated the macronuclei from Tetrahymena pyriformis GL and showed that it retained RNA polymerase activity, which catalyzed DNA-dependent -RNA synthesis. It was further demonstrated by Kurtz and Pearlman in 1972 that nuclei of



[redacted] Photomicrographs of isolated macronuclei of Tetrahymena pyriformis GL. (a) Stained with Azur C. (b) A higher maganification of (a). Stained with Azur C.

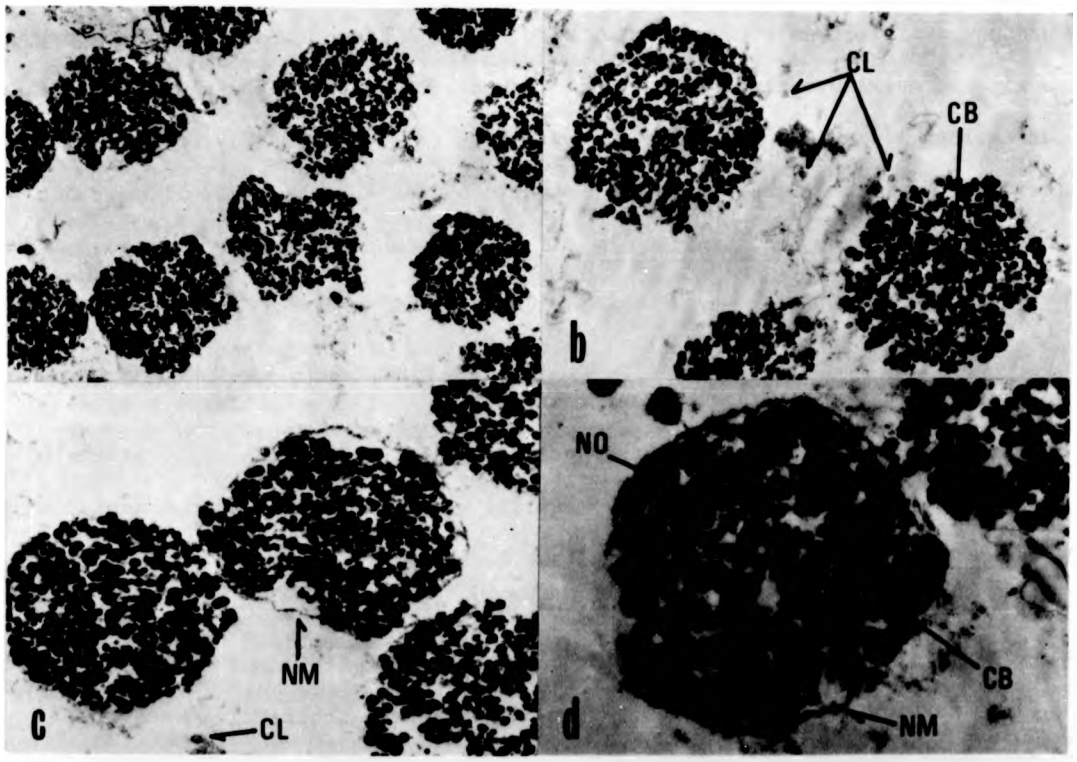


Fig. 1-15 Electron micrographs of isolated macronuclei of Tetrahymena pyriformis GL. The nuclear pellet was fixed with 3% glutaraldehyde in phosphate buffer (pH 7.4). After washing with the buffer, the specimens were postfixed with 1% OsO_4 in phosphate buffer. The specimens were then dehydrated with a series of ethanol solution and QY-1. After dehydration, the specimens were embedded in Epon 812 and cut with a Sorvall MT II-B Ultra-microtome. They were double--stained with 2% uranyl acetate and lead solution. Final samples were observed with JEM T7 electron microscope with an acceleration voltage of 60 kV.
Note the nuclear membrane is partly impaired or completely lost in most of the nuclei. Cross section of contaminating cilia is also seen. NM: nuclear membrane, CB: chromatin body, NO: nucleolus, CL: cilium. a) x3100, b) x3900, c) x5900, d) x8700.

this organism had RNA polymerase activities similar to those found in higher eukaryotes. They isolated multiple forms of RNA polymerase from sonicated nuclei and purified them by column chromatography on DEAE Sephadex. Multiple forms of DNA-dependent-RNA polymerase have also been isolated by several other workers (Higashinakagawa and Mita, 1972; Higashinakagawa et al., 1975).

In macronuclei, RNA polymerase I is responsible for ribosomal RNA synthesis and is located in nucleolus, whereas RNA polymerase II is responsible for nuclear RNA synthesis and is found in the nucleoplasm. Kumar in 1970 demonstrated that high molecular weight precursors of 25S and 17S ribosomal RNA are also synthesized in the macronuclei of Tetrahymena. Manden in 1971 found that the ribosomal RNA of this organism has a low G-C content which is lower than in higher eukaryotes. Since the ribosomal RNA accounts for about 85% of the cellular RNA, the change in the rate of net RNA synthesis during growth would directly reflect the change in ribosomes biosynthesis. The presence of amplified extrachromosomal ribosomal genes coding for 25S and 17S ribosomal RNA has been demonstrated in Tetrahymena and its palindromic structure has been studied by Karrer and Gall (1976). The development of the multiple nucleoli, 500-1000 per macronucleus, is accompanied by a several hundred-fold amplification of the ribosomal DNA gene, and is concomitant with a vigorous synthesis of ribosomal RNA and thus of ribosomes (Brown and David, 1968).

(d) Transcription control

In general, in eukaryotic cells DNA synthesis takes place within a discrete interval of the cell cycle known as the S-period. However, the synthesis of RNA and protein is a continuous process occupying the entire cell cycle (Mitchison, 1971). In different types of cell, the rate of synthesis of the three major classes of

RNA has been found to change in response to different environmental conditions. For example. starvation of Tetrahymena cells causes a dramatic decrease in RNA synthesis which is reversed on refeeding.

It is now well established that many biochemical processes are controlled through cyclic nucleotides. In Tetrahymena pyriformis the intracellular concentration of cAMP and cGMP have been measured at different stages of the cell cycle and of cell growth (Graves et al., 1976; Dickinson et al., 1976). A cyclic AMP dependent protein kinase has also been isolated and partially purified (Majumder et al., 1975).

Recently, a role for the cyclic nucleotide, cAMP and cGMP in the control of transcription has been suggested for Tetrahymena pyriformis (Nadjati-Shokouhi,/1978). It was shown that cAMP at physiological levels and dibutyryl cAMP stimulated RNA polymerase I dependent transcription in isolated macronuclei. The stimulation of endogenous RNA polymerase activity by cGMP in the presence of Ca^{++} was also reported.

1.9 Aims of the Project

The work presented in this thesis is a study of the synthesis of RNA and its control in the isolated macronuclei of Tetrahymena pyriformis.

Isolated nuclei represent a simpler system for studying transcription than the whole cell. However, they do not necessarily carry out the faithful and closely controlled transcription of the genome that one would observe in vivo, and it is important to test directly how good a model system they represent.

For this reason, the present studies had the following aims:-

(1) To check whether isolated macronuclei are capable of catalyzing all the steps of in vivo transcription or not. There are many reports in the literature of isolated nuclei not being

capable of the reinitiation of RNA synthesis. Direct tests were therefore planned to establish if Tetrahymena macronuclei were capable of reinitiation.

(2) To characterize the products of macronuclear transcription in terms of their size (sedimentation coefficients) and compare these with the values obtained for in vivo transcription products.

(3) To establish the size of the pools of free and template engaged RNA polymerase in isolated macronuclei and to relate this information to the different functional states of the enzyme. This should be a useful parameter to measure in the study of the regulation of transcription, since initiation factors might be expected to diminish the pool of free enzyme and increase the pool of engaged enzymes.

(4) To identify any putative cytoplasmic factors which might effect the rate of initiation or elongation of transcription. These factors could then be examined to see if they play any important role in the control of transcription and the exchange of information between cytoplasm and nucleus.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Biochemicals

Proteose peptone (Difco) was purchased from Difco Laboratories, Detroit, Michigan, U.S.A. Powdered yeast extract was obtained from London Analytical and Bacteriological Media Ltd., London, U.K.

The following chemical materials were purchased from Sigma Chemical Company, Ltd., calf thymus DNA (type 1: sodium salt, highly polymerized), bovine serum albumin, dithiothreitol, actinomycin D (grade I), heparin (sodium salt, grade I), 8-hydroxyquinoline and all nucleoside triphosphates. Agarose (electrophoresis grade and MES were obtained from B.D.H. Biochem. Lab. Reagents. Proteinase K was purchased from the Boehringer Corporation (London) Ltd. Poly[d(A-T)], sodium salt and total *E. coli* RNA markers were purchased from Miles Laboratories, Inc., U.S.A. NCS was purchased from Hopkins and Williams. (4-^{14}C) UTP (60 Ci/mole) was purchased from Radiochemical Centre, Amersham, U.K.

α-amanitin was kindly donated by Professor Th. Wieland, Chemical Department, Max-Planck Institute of Medical Research, Heidelberg, F.D.R. Rifamycin SV AF/013 was kindly donated by Professor L. Gii. Silvestri, Giruppo Lepetit S.P.A. Research Laboratories, Milan, Italy.

All other chemicals used were of the highest purity commercially available.

2.2 Buffers

A. 0.25 M sucrose buffer: 0.25 M sucrose, 10 mM $MgCl_2$ (pH 7.2)

B. TGMED buffer: 0.05 M Tris-HCl (pH 7.9), 30% (v/v) glycerol
3 mM magnesium acetate, 0.1 mM EDTA, 5 mM DTT

C. Electrophoresis buffer: 36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA (disodium salt) (pH 7.8) containing 0.2% SDS

D. Sample buffer: Buffer C containing 10% sucrose and bromophenol blue

E. Gel buffer: 0.16 M Tris, 0.15 M NaH_2PO_4, 5 mM EDTA (disodium salt) (pH 7.8)

F. Tris-buffer 1 mM Tris-HCl (pH 8.0)

G. Homogenizing buffer: Tris-HCl (pH 8.0), 0.5 mM DTT and 0.05 mM EDTA

H. Inorganic phosphate buffer: 0.6 g KH_2PO_4, 0.15 g K_2HPO_4 and and 0.25 g $MgSO_4$ dissolved in 1 litre distilled water and adjusted to pH 6.5 with NaOH

I. 10 mM MES buffer: 10 mM MES (pH 7.9)

J. SDS-Acetate buffer: 0.15 M Na-acetate-acetic acid (pH 6.0), 0.5% (w/v) SDS

2.3 Organism and Growth Conditions

Tetrahymena pyriformis, strain L1630/1W was obtained from the Culture Centre of Algae and Protozoa, Cambridge, U.K.

Tetrahymena pyriformis, strain W, was grown in medium containing 2% proteose-peptone (Difco), 0.1% yeast extract, 0.5% glucose and 5 µg/ml crystalline ferric chloride. The medium was made up with tap water and pH was between 7.0 and 7.2. The medium was sterilised by autoclaving at 15 lb/in^2 for 20 minutes. 400 ml cultures were grown in a 2 l Erlenmeyer flask to give high surface/volume ratio. The cultures were shaken at 140-150 oscillations/min in an orbital shaker. The temperature was maintained at 28 ± ½° C. The cultures were generally harvested between 3×10^5 - 4×10^5 cells/ml. The mean doubling time of log phase cultures was 160-180 minutes. If the cell density was allowed to reach 1.5×10^6 - 2×10^6 cells/ml the cells were in stationary phase, and immediately after that a rapid decline in

cell number followed by lysis of the cells was observed.

Stock cultures werekept statically in 100 ml medium in 250 ml conical flasks and were grown at room temperature. 1 ml of 3-4 days old stock culture was transferred twice a week to 100 ml of fresh medium under aseptic conditions. Under these conditions, a stationary phase cell density of about 5×10^5 cells/ml was obtained after 3-4 days and cells remained viable for a further 7-8 days at least.

2.4 Fixation and counting of Tetrahymena pyriformis cells

Cells were examined before counting under the microscope to assess the viability of the culture. Cells were fixed by taking an aliquot of culture and adding it to an equal volume of 20% formaldehyde neturalized with 0.01 M phosphate buffer, pH 7.2 Fixed cells were then counted in a Neubauer haemocytometer.

2.5 Isolation of Macro-nuclei

(a) Nonidet-P40 method

Tetrahymena pyriformis nuclei were isolated according to the procedures described by Mita et al., (1966) and Higashinakagawa et al., (1975). Cells grown as described in Section 2.3 were harvested at either the exponential phase or the early stationary phase. 2-3 litres of the culture were grown to give a total number of cells of about 10^9. Cells were harvested in 250 ml batches and sedimented at 5000 rpm (4080 x g) for 10 minutes in a Sorvall RC 2-B (GSA) head) at 0° C. The temperature was kept at $0\text{-}4^\circ$ C throughout the procedure. The sedimented cells were washed twice in a small volume of ice-cold sucrose buffer (A). The packed cells were then resuspended in nine volumes of the sucrose buffer. One-fifth volume of 1% Nonidet-P40 in sucrose buffer (A) was added to the cell suspension, with gentle stirring

at 0^{o} C, to give 0.16% final concentration of Nonidet-P40. At this concentration the cell membrane was disrupted and nuclei were released from the cell within 1-5 minutes. The degree of cell lysis was checked by using the light microscope and the integrity of the whole nuclei was checked by phase contrast microscopy. The cell lysate was made 2.1 M with respect to sucrose by adding solid sucrose. This was done in the cold room with stirring. The final viscous cell suspension was centrifuged at 26000 rpm (59000 x g) for one hour at 0^{o} C in an Ultra-centrifuge Spinco L5O, using the 30 rotor. The nuclei were separated from the cell debris in the 2.1 M sucrose and pelleted at the bottom of the tubes. The nuclei were gently resuspended in TGMED buffer (B) and washed twice, centrifuging at low speed, i.e. 3000 rpm (1085 x g) in a Sorval RC 2-B (S534 head). The resulting clean nuclei were resuspended in the TGMED buffer (B) to give 2 x 10^{8} nuclei/ml. The nuclear suspension was divided in 1 ml portions in small plastic vials. The vials were quick-frozen in liquid nitrogen and kept at -20^{o} C. Isolation of Tetrahymena macronuclei by this method gave approximately 80-90% recovery of the nuclei as judged by DNA estimations. The nuclei prepared by the Nonidet-P40 procedure lose their outer membrane during isolation (Higashinakagawa and Mita, 1973).

(b) Non-detergent method

This method is based on that described by Bollinger (1970).

Cells were grown and harvested as in Section 2.3. The sedimented cells were washed with 10% v/v glycerol in 10 mM MES buffer (pH 7.9) containing 2 mM $CaCl_2$ (I). The glycerol causes the cells to swell and also weakens the plasma membrane. This was clearly observed by examining the cells under the light microscope. The cells were centrifuged for 15 minutes at 10000

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rpm (12000 x g) in Sorvall RC 2-B (SS-34 head). The temperature was kept 0-4° C throughout the rest of the procedure. The cells were then resuspended in 10 volumes of 10 mM MES buffer (I) containing 2 mM $CaCl_2$ and centrifuged for 15 minutes at 10000 rpm. The resulting cell pellet was resuspended in a final volume of 10 ml in 10 mM MES buffer (I) containing 2 mM $CaCl_2$ and 0.25 M sucrose, and forced through a No. 20 hypodermic needle. This procedure causes cell breakage; the cell membrane disrupts and intact nuclei were released. After each passage through the needle, the cells were examined by light microscopy to ensure maximum cell disruption. To the homogenate was added 45 ml of 10 mM MES buffer (B) containing 2 mM $CaCl_2$ and 2.44 M sucrose. The suspension was centrifuged for 20 minutes at 13000 rpm (20000 x g) in Sorvall RC 2-B (SS-34 head). The pellet was resuspended in 10 mM MES buffer (B) containing 2 mM $CaCl_2$ and 2.44 M sucrose and centrifuged for 40 minutes at 20000 rpm in an ultracentrifuge, Spinco L-50, using a 30 rotor. The resulting nuclear pellet was suspended in 10 mM MES buffer (I) containing 2 mM $CaCl_2$ and centrifuged at 8000 rpm (7500 x g) for 10 minutes. The washed nuclei were stored as in Methods (2.1). Nuclei prepared by this method were found to be free of cytoplasmic contamination.

2.6 Isolation of Macronuclei from Starved Cells

Tetrahymena cells were starved according to the method of Cameron and Jeter (1970). Cells were grown and harvested at a cell density of 1×10^5 cells/ml from 5 litre of culture as described previously in Section 2.3. The packed cells were then washed free of growth medium with sterile inorganic phosphate buffer (H) by centrifugation and were then starved in

this buffer for 24 hours. After starvation, the cells were pelleted by low speed centrifugation and macronuclei isolated as described in Section 2.5. Clean nuclear preparations were obtained without any damage to the nuclear membrane when checked with phase contrast microscopy.

2.7 Assay of RNA Polymerase

(a) Endogenous RNA polymerase assay

RNA polymerase activity was assayed by measuring the incorporation of (4-^{14}C) UMP into acid-(TCA) precipitable material. Nuclei were assayed for total endogenous DNA-dependent RNA polymerase activity (template bound and initiated *in vivo*), using the endogenous DNA. The reaction mixture contained, in a final volume of 0.15 ml: 0.05 ml of a cocktail mixture which was composed of 7.5 µmoles of Tris-HCl, pH 7.9, 0.15 µmoles of ATP, 0.075 µmoles of each GTP and CTP, 22.5 µmoles of KCl, 0.375 µmoles of dithiothreitol, 0.225 µmoles of $MnCl_2$, 0.60 µmoles of $MgCl_2$, 0.06 µmoles of unlabelled UTP and 0.05 µCi of (4-^{14}C)UTP (60 mCi/mmole), 0.05 ml of distilled water, and the reaction was started with 0.05 ml of nuclei. After incubation for 10 minutes in a water bath at 25^{o} C, the test tubes were chilled in ice. Immediately 100 µl aliquots were charged on glass fibre discs (Whatmann, GF/C, 2.5 cm diameter) which were then immersed in ice cold solution containing 5% (w/v) TCA, 1% (w/v) sodium pyrophosphate (pH 1.5). The discs were washed three times in ice cold TCA solution (30 minutes for each wash). The discs were then washed twice in absolute ethanol and twice in diethyl ether. The discs were finally dried under an infra-red lamp and transferred to the vials containing 4 ml of toluene scintillator (5 g in PPO and 0.2 g in POPOP per litre). Radioactivity was then measured

in a liquid scintillation spectrometer, the Packard 2425 tri-carb spectrometer. Zero-time incubation blanks contained less than 0.2% of the total radioactivity, initially present in the assay mixture. Assays were performed in duplicate, and the standard error of the mean was evaluated for each experiment.

(b) Free and engaged RNA polymerase assay

The procedure used was based on that described by Yu and Feigelson (1974). RNA synthesis in isolated nuclei was measured as described in Section 2.7.a except that 30 µg of poly[d(A-T)] was added to serve as an exogenous template. In some experiments actinomycin D (25 µg) was added, together with poly[d(A-T)s to provide a measure of the free enzyme pool, under conditions where transcription of endogenous template was suppressed. The reaction was started by the addition of 50 µl nuclei to the incubation mixture in the presence of poly[d(A-T)] and actinomycin D, using the procedures already described in Section 2.7.a.

(c) Initiation and elongation assay

This assay method is based on that described by Cedar and Felsenfeld (1973) with slight modification. 0.05 ml isolated nuclei were incubated in low salt with three of the four nucleotides present at 25° C with 0.05 ml of initiation cocktail. The cocktail contained 3.75 µmoles Tris-HCl (pH 7.9), 0.112 µmoles of $MnCl_2$, 0.187 µmoles of DTT, 0.1 µmoles ATP, 0.05 µmoles of GTP, 0.015 µmoles UTP and 0.025 µCi of (4-^{14}C) UTP (sp. activity 60 Ci/mole).The initiation reaction was stopped after 15 minutes by the addition of $(NH_4)_2SO_4$ to a final concentration of 0.4 M (7.99 µmoles) or of heparin to 1 mg/ml in the presence of 22.5 µmoles KCl. This step was used to

prevent reinitiation of transcription by enzyme molecules. Elongation in high salt or in the presence of heparin was then started by the addition of 0.15 µmoles CTP and 0.75 µmoles $MgCl_2$ in a final volume of 0.15 ml of reaction mixture. After incubation for the required time in a water bath at 25° C, the test tubes were chilled in ice. Immediately 100 µl aliquots were charged on to glass fibre discs (Whatmann, GF/C 2.5 cm diameter) and the washing procedure was carried out as described in Section 2.7.a.

2.8 Extraction of RNA Synthesised in vitro

The extraction procedure used was that described by Davies and Walker (1977).

Isolated nuclei were made to synthesise radio-labelled RNA by using the first part of the standard assay procedure as described in Section 2.7.a. RNA synthesis was terminated by the addition of proteinase K (100µg/ml) at 4° C, and the mixture incubated for 30 minutes. The solution was then made 0.1% in SDS and an equal volume of a phenol-cresol organic phase (50 ml freshly distilled phenol; 0.05 g 8-hydroxyquinoline; 7 ml cresol) was added. The mixture was shaken in the cold for 3 minutes and the aqueous and organic phases were separated by centrifugation at 2000 x g for 10 minutes at 4° C. After the removal of the aqueous layer, the interface layer was suspended in 3 ml of 1 mM Tris buffer (F), and extracted at 70° C for 3 minutes with more phenol-cresol. After chilling, the aqueous phase was collected by centrifugation and combined with the first extract. The aqueous phase was re-extracted several times with phenol-cresol until the interface disappeared and RNA was precipitated at -20° C with two volumes of 95% ethanol overnight.

RNA was collected by centrifugation at 12000 x g for 20 minutes. The RNA pellet was washed with 70% ethanol containing 0.1 M NaCl and dried. The RNA was freed from contaminating phenol and detergents other than SDS, by dissolving it in acetate-SDS buffer (J) at 20° C and reprecipitated with 2 volumes of 95% ethanol at -20° C. The RNA was stored at -20° C until required for electrophoresis.

2.9 Gel Electrophoresis of Labelled Nuclear RNA Synthesised in vitro

The procedure used is based on that described by Peacock and Dingman (1968), but using the electrophoresis buffer described by Loening (1967).

(a) Preparation of gels

The method of Ringborg et al. (1970) was followed with slight modification.

Agarose-acrylamide composite gels:

A stock solution of 15% acrylamide and 0.75% bis-acrylamide was prepared in distilled water after previous recrystallisation with chloroform and acetone. 0.5% agarose-2.5% acrylamide composite gels were prepared by heating 0.075 g agarose in 9.5 ml of distilled water and, after dissolution, lowering the temperature to 35° C in a water bath. 2.5 ml acrylamide-bisacrylamide stock solution was mixed with 3 ml of gel buffer (E) and deaerated under vacuum for 30 secs. The solution was then warmed at 35° C and mixed with the agarose solution. 25 µl of TEMED (N,N,N',N'-tetramethylethyldienediamine) and 0.25 ml of freshly prepared aqueous 10% ammonium persulphate were added, and the solution was gently mixed. It was then immediately pipetted into plexiglass tubes to a height of 9 cm. The columns were kept at room

temperature for 20 minutes for the completion of the polymerisation reaction before electrophoresis.

(b) Electrophoresis

Vertical tube equipment was used. The tubes are placed in the electrophoresis unit and 250 volumes of electrophoresis buffer (C) are added to each electrolyte container. The gels were pre-run at 5 m A/gel for approximately 30 min to remove free acrylamide, catalyst and other impurities. The RNA samples were dissolved in sample buffer (D) and 25 µl samples layered on the gel without removing the electrolyte buffer. Electrophoresis was continued at 5 m A/gel for 2 hrs at room temperature.

(c) Analysis of polyacrylamide gels

(i) Measurement of ultra-violet absorption of RNA

After electrophoresis, the gels were made to slide out of the electrophoresis tubes into test tubes containing distilled water. The water was replaced several times over a period of 30 minutes to wash out the high u.v. absorbing materials which accumulated at both ends during electrophoresis. The gels were then placed in a 1 cm light-path cuvette and scanned at 260 nm in a Gilford-2000 spectrophotometer fitted with a 2410-S linear transport scanning attachment.

(ii) Measurement of radioactivity of RNA

The sample gels were sliced into between fifty to sixty discs. The slicer used was made of razor blades arranged in a parallel array with spaces of 1.5 mm between blades. The slices were transferred to scintillation vials containing 10 ml of toluene scintillator (5 g PPO, 0.5 g dimethyl POPOP and 50 ml NCS per litre). The gel slices dissolved in this mixture when kept overnight at room temperature. The samples were counted in

a liquid scintillant spectrophotometer (Packard 2425 tri-carb).

2.10 Preparation of Cytoplasmic extracts

(a) Chick embryo cytoplasmic extract

Eggs were incubated at 37° C under humid conditions. After 7 days, eggs were broken and excess fluid was strained out. Embryos of reasonable size were removed to a glass petri dish. They were then cleaned from extraneous material and excess of fluid was blotted on Whatman 3 mM filter paper. The embryos were homogenized in ice with a small volume of homogenized buffer (G), using a glass homogenizer. The homogenate was then centrifuged at 2500 x g for 30 minutes at 4° C. The clear supernatant was removed, avoiding the lipid, and recentrifuged at 70000 x g for 30 minutes at 4° C. The protein concentration was adjusted to 10 mg/ml using homogenizing buffer, and extract was stored in aliquots at -70° C.

(b) Xenopus oocyte cytoplasmic extract

Extracts were prepared according to the method described by Crampton and Woodland (1979a) with slight modification. Freshly prepared oocytes from Xenopus laevis were transferred to a glass homogenizer and homogenized gently in a few ml of buffer (G). The homogenate was then centrifuged at 2500 x g max for 30 min at 4° C. The clear supernatant was removed, avoiding the lipid, and recentrifuged at 70000 x g for 30 min at 4° C. The supernatant was stored at -70° C.

(c) Tetrahymena pyriformis cytoplasmic extract

Cells were grown as described in Section 2.3, and were pelleted at the early exponential phase (2 x 10^5 cells/ml) or stationary phase (1 x 10^6 cells/ml). The cells were then washed with a small volume of homogenizing buffer (G), resuspended in

2 volumes of the same buffer and homogenized. The homogenate was then centrifuged at 1200 x g for 10 min at 4°C and the supernatant recentrifuged at 105000 x g for 1 hr at 4°C. The resulting clear supernatant was then stored in aliquots at -70° C.

2.11 DNA determination in macronuclei

(a) Extraction of DNA from nuclei

Nuclear samples were deproteinized with 5% (w/v) cold trichloroacetic acid (TCA). The TCA precipitatable material was collected by centrifugation and the supernatant discarded. DNA was extracted from the pellet by heating to 70° C for 15 minutes in the presence of 0.5 M perchloric acid. After centrifugation, the pellet was extracted again and the supernatant from both extraction steps was combined and used for DNA assay

(b) DNA assay

Nuclear DNA in the extracted samples was determined according to the method of Burton (1956; 1968). This DNA assay is based on the reaction between the deoxyribose residues of DNA and diphenylamine in the mixture of acetic acid and sulphuric acid. Calf thymus DNA was used to construct the standard curve which was linear for samples containing 0-60 µg DNA.

2.12 Protein Determination

Protein was estimated by the method of Lowry et al. (1951). A standard curve was constructed, using crystalline bovine serum albumin, to give a range of between 10-100 µg protein.

2.13 RNA Determination

RNA was determined by measuring the A_{260} of the solution. A 10 µg/ml solution was taken to have an A_{260}^{1cm} of 0.2.

CHAPTER 3

EFFECTS OF INITIATION INHIBITORS ON IN VITRO RNA SYNTHESIS

3.1 Introduction

In the study of the control of transcription in eukaryotes, one is seeking to define the regulatory components involved in gene expression and a prior knowledge of the basic mechanism of transcription is essential. Attempts have been made to define the regulatory elements of transcription with subcellular components such as (a) purified enzymes, (b) isolated chromatin and (c) isolated nuclei. Initiation and elongation steps of RNA synthesis can be separated and measured independently by a number of different methods. One method is to use either rifamycin AF/013 or heparin, which inhibit initiation but permit elongation. Another method is to select a salt concentration and combination of nucleotides which allows only initiation to occur, while other selected conditions allow only elongation to proceed in the absence of new initiation.

Cedar and Felsenfeld (1973) have studied the transcription of chromatin under conditions where the initiation step in the polymerase reaction can be separated from the propagation step. They showed that the binding of RNA polymerase to template occurred at low salt concentrations with three of the four of the requisite nucleotides present. Further initiation was inhibited by the presence of high salt, i.e. when ammonium sulphatewas added to the reaction mixture to a final concentration of 0.4 M. RNA chains already initiated could, however, be elongated when a fourth nucleoside triphosphate was added.

This method has proved useful in separating the initiation and elongation steps of transcription, and has been used by many research workers. Specific inhibitors of bacterial RNA

polymerase have been used to determine the role of the prokaryotic polymerase in RNA synthesis. The discovery of the rifampicin which binds specifically to the bacterial enzyme (Wehrli et al., 1968) made it possible to characterize the series of events which precede the initiation step of transcription. This drug inhibits the preinitiation phase by affecting the primary binding of free enzyme to DNA. While rifampicin is without influence on the RNA polymerases of eukaryotic origin, semisynthetic derivatives of the drug, affecting these enzymes have been developed (Sethi and Okano, 1976). Among these derivatives, rifamycin AF/013 has been shown to be a potent inhibitor of eukaryotic RNA polymerases. The mechanism of inhibition is more complex than the mode of action of rifampicin on the bacterial enzyme. They inhibit both the primary binding step and the temperature dependent step which is related to the opening of the two DNA strands over a short local region at the initiation site.

Other studies designed to investigate the mechanism of transcription have been done using heparin. The polyanion heparin binds tightly to free RNA polymerase molecules, and effectively inhibits initiation but not the elongation of RNA chains by either prokaryotic or eukaryotic RNA polymerases (Zilling et al., 1970; Cox, 1973). This polyanion can be used to study the elongation of RNA chains already initiated in vivo. It differs from rifamycin AF/013 in stimulating the rate of RNA synthesis (Hentschel and Tata, 1978), because it causes decondensation of inert chromatin (Hildebrand et al.,1977).

In this chapter, a study is described using isolated nuclei from Tetrahymena pyriformis. A variety of methods have been used to investigate if these isolated nuclei are capable of initiating

the transcription of new RNA chains in the absence of any factors isolated from the cytoplasm.

3.2 Results

(a) Effect of heparin concentration on RNA synthesis in isolated nuclei

The results shown in Fig. 3.1 indicate that the addition of heparin to isolated nuclei in vitro does not inhibit, but in contrast stimulates RNA synthesis. RNA synthesis gradually increases with increasing concentrations of heparin until a plateau is formed at concentrations greater than 1 mg/ml.

(b) RNA synthesis in isolated nuclei in the presence and absence of heparin as a function of time

The transcription of endogenous DNA in isolated nuclei has been shown to be stimulated by the addition of heparin in fixed time assays (Fig.3.1). The results in Fig.3.2 show that RNA synthesis in isolated nuclei increases linearly with time. When heparin is added at a concentration of 1 mg/ml to the assay mixture, the rate of RNA synthesis is greater than in the control experiment, but is less linear with time. Because of this effect, the extent of stimulation produced by heparin over the control decreases slightly with time.

(c) RNA synthesis in isolated nuclei in the presence of high salt (0.4 M $(NH_4)_2SO_4$

In order to check whether nuclei are capable of initiating RNA chains, assay conditions were chosen which separate the initiation from the elongation steps. In low salt, nuclei exhibit a very low rate of incorporation of labelled UTP in the presence of the three ribonucleoside triphosphates (ATP, GTP, cold and labelled UTP). After 15 minutes, ammonium sulphate was added to

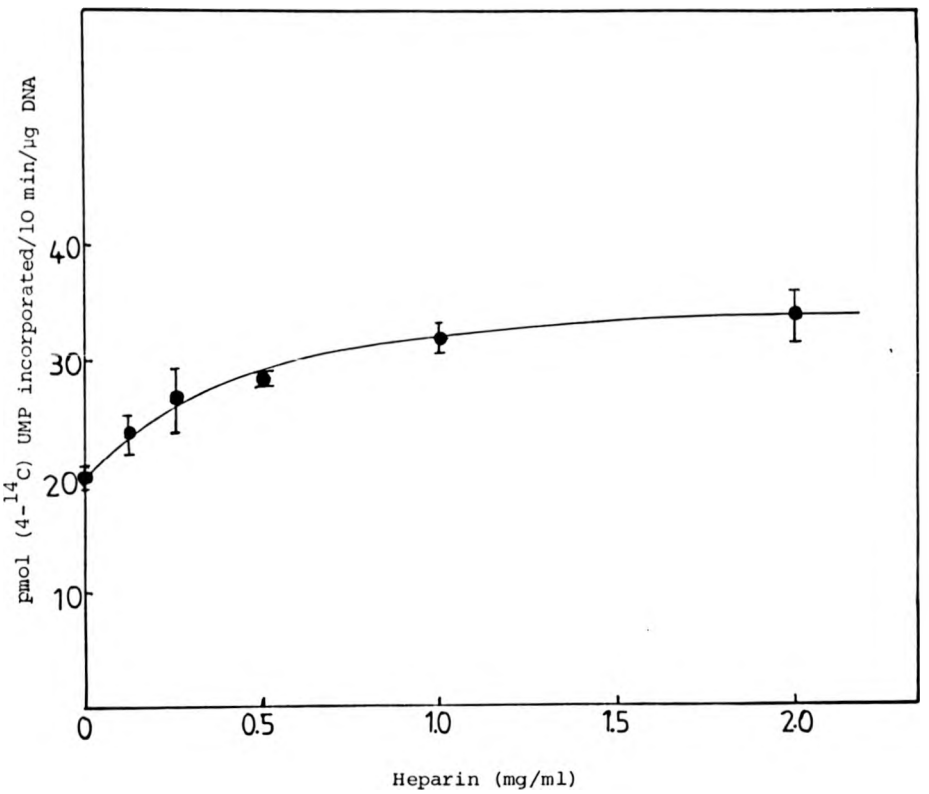


Fig. 3.1 The effect of heparin on RNA synthesis in isolated nuclei

Nuclei were isolated from exponential cells (3 x 10^5 cells/ml) and assayed for endogenous RNA polymerase activity as described in Section 2.7 (a).

Heparin was added to the incubation mixture at the concentration indicated in the figure. The reaction was started by the addition of nuclei containing 40 µg/DNA.

[(n = 2), n = number of experiments performed]

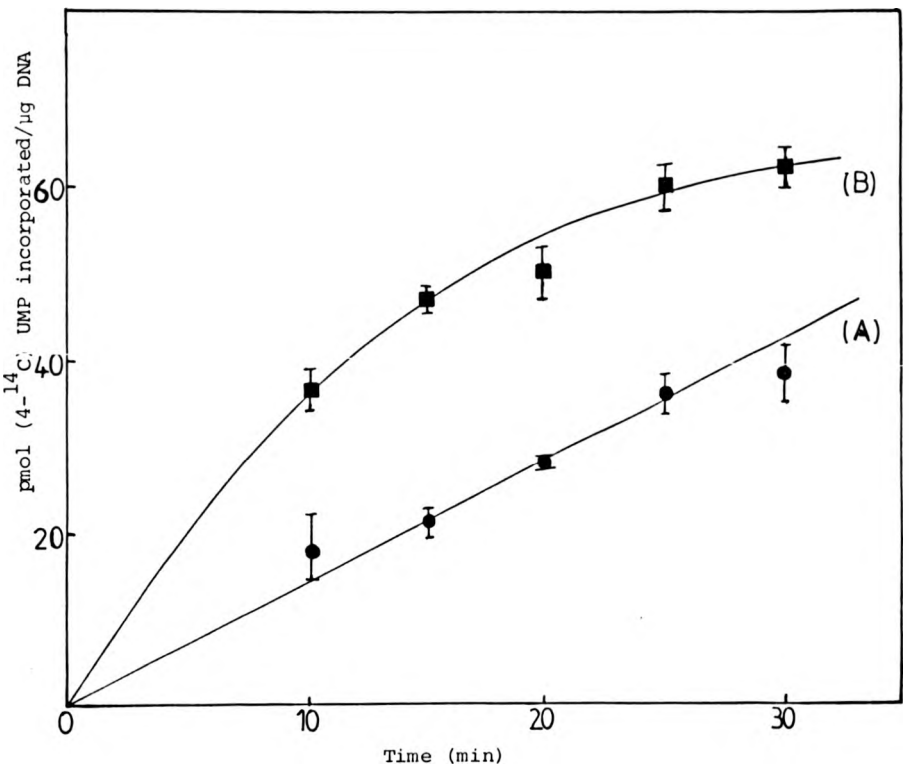


Fig. 3.2 RNA synthesis in isolated nuclei in the presence and absence of heparin as a function of time

Nuclei were isolated from exponential cells as described in Section 2.5 (a) and assayed for endogenous RNA polymerase activity as described in Section 2.7 (a). Nuclei containing 40 µg DNA were used per assay. Curve A (-●—●-) represents the RNA synthesis in the absence of heparin Curve B (-■—■-) represents the RNA synthesis in the presence of heparin (1 mg/ml).

[(n = 2), n = number of experiments performed]

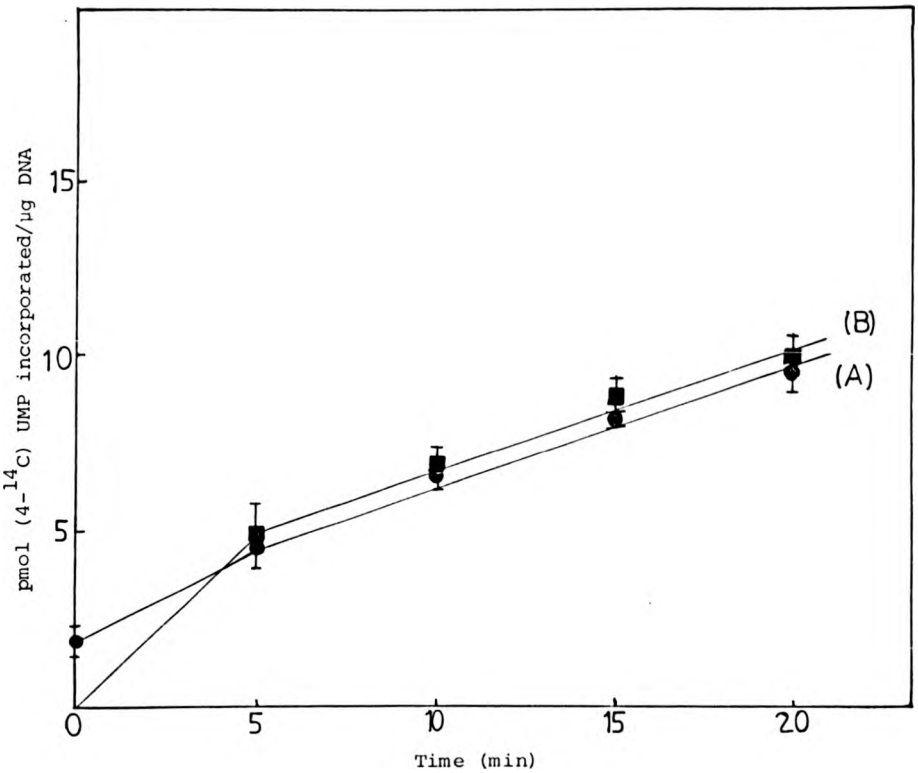


Fig. 3.3 RNA synthesis in isolated nuclei in the presence of high salt (0.4 M $(NH_4)_2SO_4$)

Nuclei were isolated from exponential cells as described in Section 2.3. RNA synthesis was measured according to the assay method described in Section 2.7(c). Nuclei containing 35 µg DNA were used per assay.

Curve A (-●-●-) RNA synthesis in high salt (0.4 M $(NH_4)_2SO_4$) following preincubation of nuclei with ATP, GTP, UTP and labelled UTP in low salt for 15 minutes

Curve B (-■-■-) RNA synthesis in high salt (0.4 M $(NH_4)_2SO_4$) without preincubation

[(n = 2), n = number of experiments performed]

a final concentration of 0.4 M, and RNA synthesis in high salt was started by the addition of the fourth nucleotide (CTP). The RNA synthesis observed (curve A) was a measure of elongation in the absence of any new initiations, apart from those which have already occurred in vivo and those which might have occurred during the preincubation. In a control experiment (curve B), the elongation of RNA synthesis was measured without the 15 minute preincubation step, so that only those RNA chains initiated in vivo could be elongated. As shown in Fig.3.3, RNA synthesis proceeds at virtually the same rate in these two experiments. Therefore we conclude tentatively that no new initiations have occurred in the preincubation period.

(d) RNA synthesis in isolated nuclei in the presence of heparin

As heparin is known to inhibit initiation but not elongation, it has been used together with 0.15 M KCl to replace the high salt treatment of the last experiment and to validate the tentative conclusion given above. Curve A in Fig.3.4 shows the elongation phase of RNA synthesis after a 15 minute preincubation in the absence of heparin under conditions which would have favoured new initiations. Curve B shows the elongation phase of RNA synthesis without such a preincubation step. Since the two curves are virtually identical, it can be concluded that no new initiations have taken place.

(e) RNA synthesis in isolated nuclei in the presence and absence of rifamycin AF/013

Fig. 3.5 shows the rate of RNA synthesis in the presence (●) and absence (■) of rifamycin AF/013. As the curves are virtually identical, it can again be concluded that isolated nuclei under standard assay conditions are only capable of RNA elongation synthesis. In this experiment, the control activity was measured

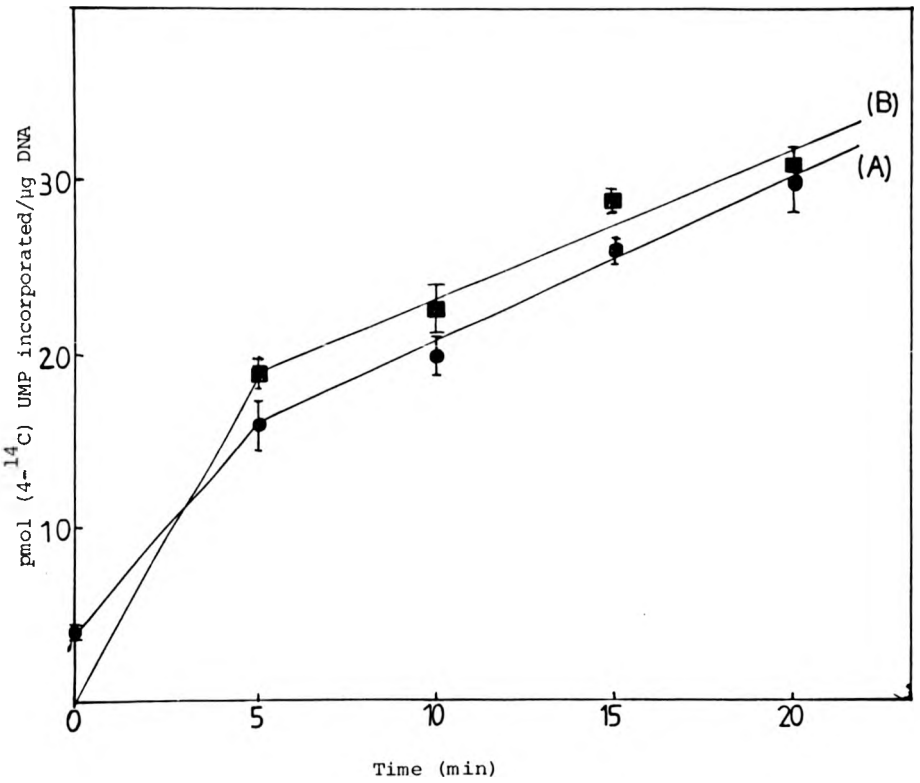


Fig.3.4 RNA synthesis in isolated nuclei in the presence of heparin

Nuclei were isolated from exponential cells as described in Section 2.5(a). RNA synthesis was measured according to the assay method described in Section 2.7(c).

Curve A (●—●) RNA synthesis in the presence of heparin and 0.15 M KCl, following preincubation of nuclei with ATP, GTP, UTP and labelled UTP in low salt for 15 minutes.

Curve B (■—■) RNA synthesis in the presence of heparin and 0.15 M KCl without preincubation.

The concentration of heparin used in the experiment was 1 mg/ml.

[(n = 2), n = number of experiments performed]

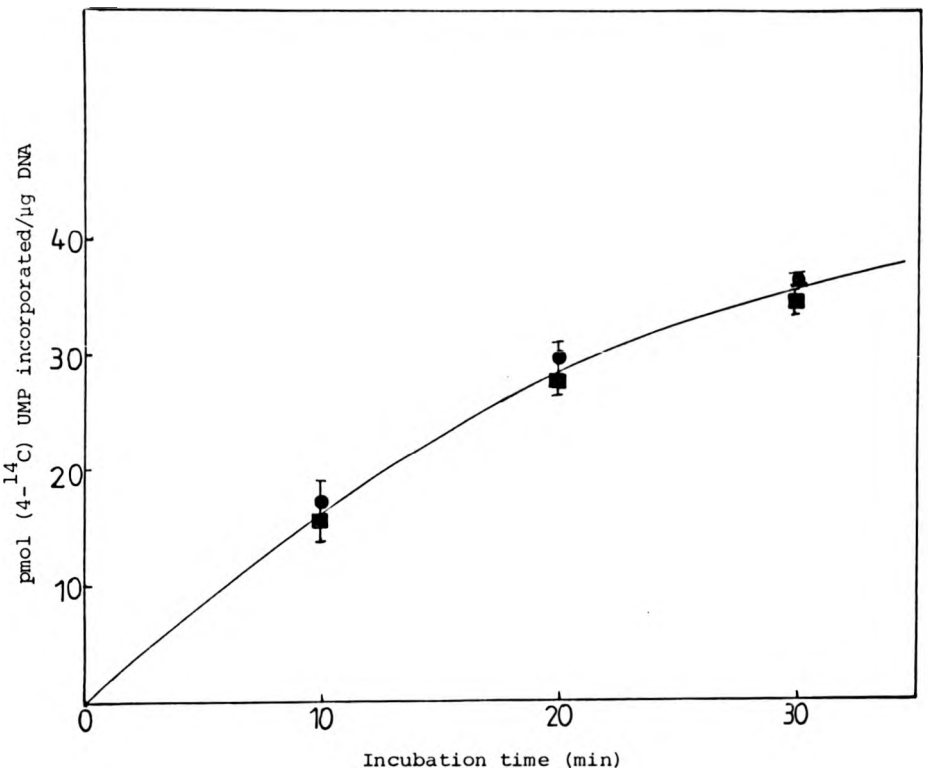


Fig.3.5 RNA synthesis in isolated nuclei in the presence and absence of rifamycin AF/013

Nuclei were isolated from exponential cells as described in Section 2.5(a)and assayed for endogenous RNA polymerase activity as described in Section 2.7(a). Nuclei containing 40 µg DNA were used per assay.

(-■-) RNA synthesis in the presence of 10 µl of dimethylsulphoxide.

(-●-) RNA synthesis in the presence of 10 µl of rifamycin AF/013 (60 µg/ml in dimethylsulphoxide).

[(n = 2), n = number of experiments performed]

in the presence of dimethyl sulphoxide, the solvent for rifamycin AF/013, which itself shows a slight inhibitory effect on RNA synthesis.

(f) The effect of preincubating isolated nuclei with heparin or rifamycin AF/013

The results of the last two experiments have been confirmed as shown in Table 3.1 in a set of separate experiments in which RNA synthesis was allowed to proceed for a fixed time (10 minutes), with or without preincubation with heparin and rifamycin AF/013.

3.3 Discussion

It is generally accepted that the *in vitro* RNA synthesis of isolated nuclei and chromatin involves the elongation of pre-existing chains (Marzluff *et al.*, 1973), and that negligible reinitiation of the endogenous template usually occurs (Cox, 1976). In this chapter, this hypothesis has been examined and confirmed in the case of *Tetrahymena pyriformis* nuclei by a variety of different techniques. The results have shown that the addition of heparin to isolated nuclei *in vitro* does not inhibit but, in contrast, stimulates the RNA synthesis. A similar result was obtained by Ferencz and Seifart (1975) who studied RNA synthesis in isolated rat liver nucleoli. Heparin is known to cause morphol ogical alterations of nuclei, probably related to its anionic properties, which lead to the dissociation of DNA associated proteins in chromatin (Scheintaub and Fiel, 1973). This effect could easily contribute towards enhanced RNA synthesis by physically facilitating the transcription process. The mechanism of action of heparin has been further discussed by Coupar and Chesterton (1977). They showed that the stimulation of RNA synthesis involves only polynucleotide elongation rates, and that

Table 3.1 The effect of preincubation of nuclei with heparin or rifamycin AF/013 on RNA synthesis

	Incubation conditions	RNA polymerase activity (pmol of [4-^{14}C] UMP incorporated/10 min/ µg DNA)
1.	Control	19 ± 0.1
2.	+Dimethylsulphoxide (10 µl)	17.5 ± 0.8
3.	+Heparin (1 mg/ml)	30.0 ± 1
4.	+Heparin (preincubated with nuclei)	29.7 ± 2
5.	+Rifamycin AF/013 (60 µg/ml)	18.0 ± 1.2
6.	+Rifamycin (preincubated with nuclei)	18.2 ± 1

Nuclei (35 µg DNA per assay) were isolated from exponential cells and assayed forendogenous RNA polymerase activity as described in Section 2.7 (a). Where indicated, nuclei were preincubated with heparin or rifamycin AF/013 for 15 minutes at 25° C. The reaction was started by the addition of complete assay cocktail to the nuclei, and the RNA synthesized in a 10 minute incubation period was measured.

[(n = 2), n = number of experiments performed]

the free enzyme was inhibited and prevented from initiating on the template.

The results reported here on using selected salt concentrations and combinations of nucleotides to favour either initiation or elongation need further discussion. Preincubation in a system lacking one nucleoside triphosphate can block chain propagation, a technique applied recently to the transcriptional studies by Sarkander and Uthoff (1976). Figs.3.3 and 3.4 show that isolated nuclei preincubated with three nucleotides allow a very limited incorporation of label into RNA. This may be explained either by a trace contamination of the nucleoside triphosphates with CTP or an endogenous pool of nuclear CTP (Hyman and Davidson, 1970). A small amount of incorporation could also be due to slight elongation of already initiated oligonucleotides. The use of high salt (0.4 M ammonium sulphate) inhibits the reinitiation but also dissociates chromosomal proteins from DNA (Pogo et al., 1967). It has also been show that all three types of RNA polymerase activity were progressively inhibited by increasing $(NH_4)_2SO_4$ concentration (Higashinakagawa et al. (1975). The use of heparin in the presence of low salt concentrations (0.15 M KCl) was therefore introduced in the study of elongation synthesis to avoid any artefacts associated with the use of high salts. However, heparin itself has a stimulatory effect on elongation, even though reinitiation is not taking place. This problem was overcome by making use of appropriate controls to confirm that RNA synthesis in isolated nuclei was due to propagation of RNA chains already initiated in vivo. Fig.3.5 shows the absence of any effect of rifamycin AF/013 on the RNA synthesis in the isolated nuclei from Tetrahymena pyriformis.

The concentration of rifamycin, 60 µg/ml, used in this experiment was specifically chosen to inhibit initiation, since a very high concentration (400 µg/ml) is required to inhibit elongation (Juhasz et al., 1972). Table 3.1 shows that the preincubation of Tetrahymena nuclei with initiation inhibitors does not effect RNA synthesis as compared with appropriate controls. In general, similar results have been obtained with most in vitro RNA synthesizing systems from other eukaryotes.

Due to the presence of a hydrophobic side chain in the structure of rifamycin AF/013, dimethyl sulphoxide was used as a solvent for this compoumd. The slight inhibition of RNA synthesis by dimethyl sulphoxide itself may be due to its denaturing effects on macromolecules, such as DNA and chromosomal proteins (Herskovitz, 1962). Mitryaev and Belous (1978) have studied the effect of dimethyl sulphoxide on RNA synthesis, using isolated rat liver nuclei. They showed that the DNA-dependent RNA polymerase activity was inhibited at concentrations above 10% dimethyl sulphoxide. In the experiment reported here, a final concentration of 6% was used in the assay mixture.

CHAPTER 4

STUDIES ON THE SIZE DISTRIBUTION OF IN VITRO RNA PRODUCTS FROM ISOLATED MACRONUCLEI

4.1 Introduction

It is now well established that in eukaryotes all RNA species are synthesized *in vivo* in the forms of precursors of high molecular weight. During maturation (post transcriptional processing), the precursor molecules are cleaved, most probably with specific endo- or exonucleases into defined intermediates which have distinct sizes and functions. To study the transcription in a cell free system, the question arises as to the extent to which this process can be mimicked *in vitro* when the transcribing machinery and defined templates are used. One of the basic criteria to establish the fidelity of transcription is to characterize the RNA species synthesized *in vitro*. The RNA species formed can be easily characterized on the basis of their nucleotide composition, hydridization behaviour and sedimentation pattern.

Despite the limited RNA synthesis found in isolated nuclei, it is possible to determine the sizes of transcripts made *in vitro*. Marzluff and Huang (1975) have showed that a system of isolated mouse myeloma nuclei appeared to fulfil most of the conditions required for faithful transcription. Studies on transcription, using isolated nuclei from *Dicytostelium discoideum* showed that the nuclei were capable of elongating and terminating the transcripts apparently correctly; thus the size distribution of transcripts resembled that *in vivo* (Jacobson *et al.*, 1974). The combined use of the different techniques of phenol extraction and sedimentation analysis of isolated RNA fractions permits a considerable fractionation of the main components of newly formed cellular RNA. The procedures for isolation, purification and fractionation of RNA

have been discussed in detail by Poulson (1973). The RNA chains can be specifically labelled by providing α-^{32}P-NTP or ^{3}H- or ^{14}C-labelled ribonucleoside triphosphates in the reaction medium and the RNA formed can be isolated by either hot or cold phenol treatment.

Tetrahymena pyriformis contains ribonuclease activity, which is responsible for the degradation of nuclear RNA, both in vivo and in vitro (Lazarus and Scherbaum, 1967). The cold phenol-Tris-SDS procedure described by Hellung-larsen et al. (1971) has been used to extract RNA from whole cells or nuclei of Tetrahymena. The use of SDS and extraction at low temperature minimizes the degradation of RNA, which can occur during the initial stages of extraction. Ribonuclease inhibitors are also often used to minimize the degradation of isolated RNA. Mendelson and Young (1978) showed that the combination of SDS with a powerful proteolytic enzyme such as proteinase K also stopped the degradation of RNA. The efficiency of RNA extraction by the phenol/SDS procedure using proteinase K has been discussed by Davies and Walker (1978). This technique has been adopted in the work described in this Chapter.

Analysis of the size distribution of RNA synthesized in vivo or in vitro can be made by separating the isolated RNA on sucrose gradients by zone centrifugation techniques or by polyacrylamide gel electrophoresis. Fractionation of RNA by gel electrophoresis is more rapid and produces better resolution than sucrose gradient centrifugation (Loening, 1967). In the work reported here, polyacrylamide-agarose composite gel electrophoresis was used to fractionate the labelled RNA products made by isolated macronuclei (Niles, 1978).

4.2 Results

(a) Gel electrophoretic analysis of RNA synthesised in isolated macronuclei after 10 minutes

The electrophoretic analysis of nuclear RNA synthesised in

vitro in *Tetrahymena* is given in Fig. 4.1. The RNA products synthesised *in vitro* were isolated from macronuclei as described in Section (2.8) and analysed by acrylamide-agarose gel electrophoresis. This method provides a means of monitoring both high and low molecular weight macromolecules (RNA) simultaneously with high resolution. The profile of RNA transcripts after incubation of macronuclei with substrates for 10 minutes showed a heterogeneous population, but five distinct peaks could be observed. Since the electrophoretic mobilities of RNA are inversely proportional to the sedimentation coefficient (Peacock and Dingman, 1968), and there is an approximately linear relationship between the log of the molecular weight of an RNA molecule and the relative distance it migrates in a particular gel, it was possible to predict M.W/S values of individual peaks using markers of known S value. The markers used were *E. coli* RNAs (23S, 16S and 4S) and *Xenopus* ribosomal RNA (28S and 18S) which were run on the gel under identical conditions to that used for analysing isolated labelled nuclear RNA samples.

As can be seen from Fig. 4.1, some of the products of RNA synthesis could be distinguished as specific peaks on gel electrophoresis. Distinct peaks occurred at approximately 25S and 17S, the sizes of fully processed ribosomal RNA seen in *in vivo* experiments. A small peak with a sedimentation coefficient between 4S and 5S was also observed, and this may represent newly synthesised 5S ribosomal RNA or possibly tRNA. In addition, two other minor components at 21S and 15S were seen. However, no 45S RNA (pre-ribosomal RNA) was detected, which indicates a very rapid turnover rate of these unstable large precursor molecules that might be synthesised in isolated macronuclei during a 10 minute incubation.

Figure 4.1 and Figure 4.2

Electrophoretic separation of labelled RNA products synthesised by isolated macronuclei in vitro

Macronuclei were isolated from exponentially growing Tetrahymena cells as described in Section (2.5.a). Isolated macronuclei were incubated with the assay cocktail as described in Section (2.7.a) except that the amounts were scaled up 3-fold. The RNA synthesised in a 10 minute period was extracted from 0.45 ml of incubation mixture by the phenol-SDS extraction method, described in Section (2.8). The extracted labelled nuclear RNA was run on 2.5% acrylamide - 0.5% agarose gel as described in Section (2.9.b). After the end of electrophoretic run, gels were sliced into 1.5 mm slices and counted for radioactivity as described in Section (2.9.c.i). Unlabelled E. coli total RNA (23S, 16S and 4S) and Xenopus oocyte ribosomal RNA (28S and 18S) were run separately under the same experimental condition. The position of these marker RNA species is indicated by arrows on the Figure. They were located in the gel by absorbance measurements at 260 nm, as described in Section (2.9.c.ii).

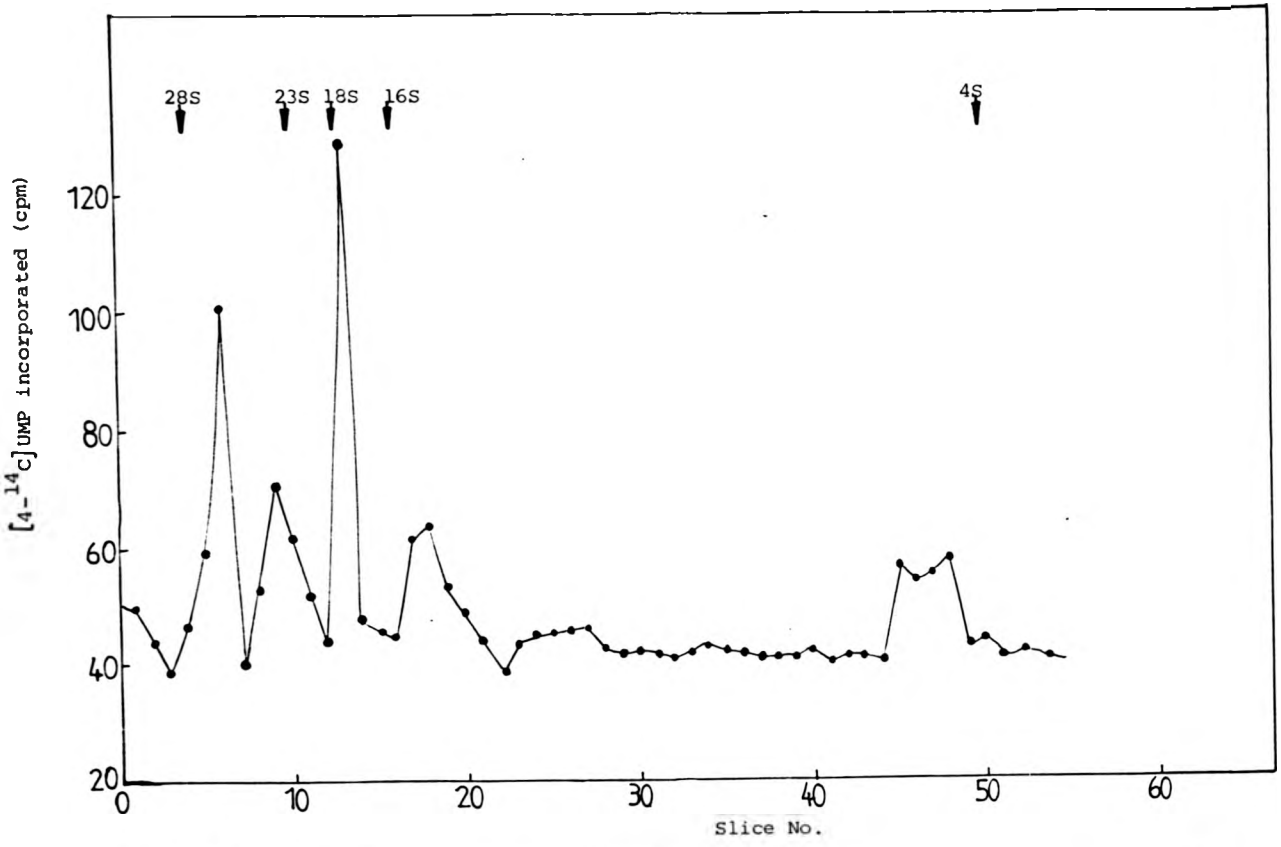


Figure 4.1 Gel electrophoretic profile of nuclear RNA synthesised in vitro in the absence of heparin

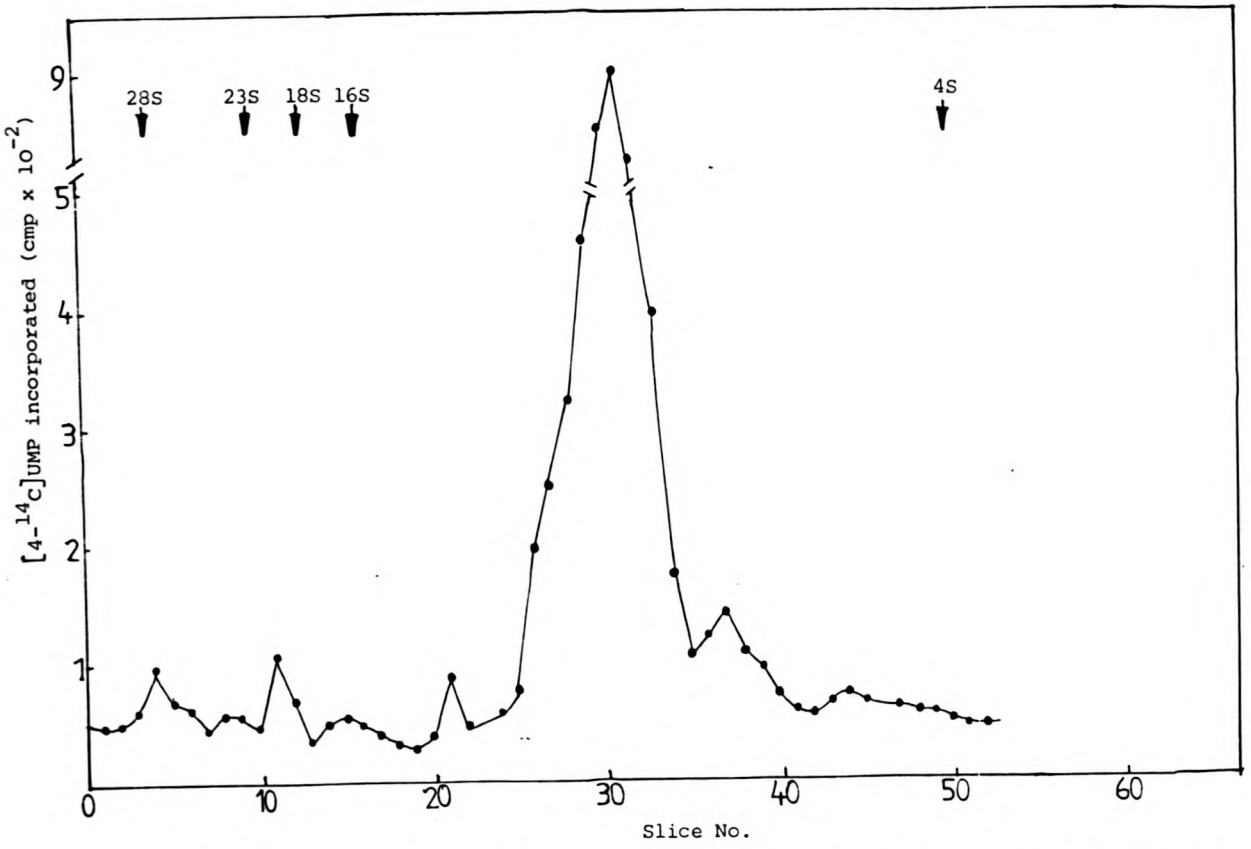


Figure 4.2 Gel electrophoretic profile of nuclear RNA synthesised in vitro in the presence of heparin

(b) Gel electrophoretic analysis of RNA synthesised in the presence of heparin in isolated macronuclei

The gel electrophoretic profile of labelled nuclear RNA synthesised in the presence of heparin showed dramatic differences in the size distribution pattern when compared to a control without heparin (Fig. 4.2). In the presence of heparin, the major product, about 60% of all the RNA synthesised, was a broad symmetric peak with a sedimentation coefficient of 9S. This peak was not seen in the absence of heparin. Two other peaks were observed with sedimentation coefficents at 28S and 20S. but these correspond to less than 10% of the total RNA synthesised. In addition, small components at 11S, 6S and 5S were also seen. In the presence of heparin, the large precursor rRNA (45S) was not seen, although the two peaks at 28S and 20S may correspond to partially processed rRNA. In the electrophoretic profile in the absence of heparin, two peaks containing approximately the same amount of RNA were seen at 25S and 17S. These could correspond to fully processed rRNA, but more definitive characterisation is required. However, the sizes of the major rRNA species produced in cells under in vivo conditions are also 25S and 17S.

(c) Studies on the release of newly synthesised RNA from isolated macronuclei

Table 4.1 shows both the total incorporation of [^{14}C]-UMP into RNA by isolated macronuclei and the radioactivity incorporated when the nuclear pellet was separated from the supernatant after a 10 minute incubation with substrates. The results show that about 15% of the total incorporation was released from the macronuclei during the incubation period. Most of the newly synthesised RNA (85%) was still associated with the nuclear pellet.

Table 4.1

Release of newly synthesised RNA from isolated macronuclei

Experimental conditions		$[4\text{-}^{14}C]$-UMP incorporated (CPM)
1. Macronuclei		697 ± 62
2. Macronuclei	Supernatant	110 ± 10
	Nuclear pellet	594 ± 20

Macronuclei were isolated from exponentially growing cells as described in Section (2.5.a). Macronuclei containing 35 µg DNA were incubated with reaction mixture for 10 minutes at 25° C, and the total RNA synthesis was measured according to the method described in Section (2.7.a). In a second experiment at the end of a 10 minute incubation period, macronuclei were separated from the incubation media by centrifugation at 500 x g. The resulting pellet and supernatant were counted separately to give an estimate of the newly synthesised RNA.

[(n = 2), n = number of experiments performed]

4.3 Discussion

The present study is an analysis of the size distribution of RNA products synthesised in isolated macronuclei of Tetrahymena pyriformis, using SDS gel electrophoresis. The results described show that synthesis of different classes of RNA is occurring in isolated macronuclei in vitro and the major RNA species seen are 25S and 17S. The sizes of these RNA species synthesised in in vitro experiments are comparable to those obtained for rRNA in in vivo studies by Leick (1969), who used exponentially growing cells of Tetrahymena pyriformis strain GL. In Leick's experiments, cells were incubated with ^{3}H -uridine and RNA was extracted from macronuclei by the phenol-SDS method. In these in vivo studies, evidence was obtained for the synthesis of 25S and 17S rRNA, but short ^{3}H -uridine pulse experiments showed that rRNA precursors with sedimentation coefficients of 45S and 32S were initially produced but rapidly processed to the 25S and 17S RNA species. Furthermore it was shown that whole cells synthesise much less mRNA (< 20% of the total) compared to rRNA and its precursors. From in vivo experiments, mRNA is usually located in the 8-15S region. The putative mRNA synthesised by isolated macronuclei does not appear to constitute very much of the total RNA synthesised since no major peak is observed in the 8-15S region except for a minor peak seen at 15S.

In the presence of heparin, a dramatic stimulation of putative mRNA synthesis (8-15S) is evident. This type of stimulation has also been reported for rat liver by Coupar and Chesterton (1977). They deduced that the stimulation was associated with an increased rate of elongation of RNA chains by RNA polymerase II. They were also able to demonstrate that the effects of heparin were not due to inhibition of nuclear ribonucleases. The stimulation of elongation

rates could be explained in terms of activation of RNA polymerase molecules already bound to the chromatin complex, perhaps by a derepression mechanism. It is known that heparin can complex with histones and that histones can repress transcription.

One of the major deductions from the work presented here is that transcription products can be produced *in vitro* by isolated macronuclei which have the same size (or S value) as RNA synthesised *in vivo*. Thus 17S and 25S RNA molecules are synthesised both in *in vitro* and *in vivo* experiments. The transcription system in isolated macronuclei is therefore probably capable of terminating RNA chains correctly.

The processing and transport out of the nucleus of newly synthesised RNA will be discussed in Chapter 7.

CHAPTER 5

FREE AND ENGAGED FORMS OF RNA POLYMERASE I AND II IN ISOLATED MACRONUCLEI

5.1 Introduction

Changes in environmental and growth conditions can produce large changes in the rate of RNA synthesis in Tetrahymena cells, particularly in ribosomal RNA synthesis. In this chapter evidence is presented for the existence of pools of free and engaged forms of RNA polymerase in Tetrahymena macronuclei. The amount of template engaged enzyme correlates with the general level of RNA synthesis and factors which control the pool size of the two different forms of RNA polymerase must be involved in the overall regulation of transcription. The amount of free RNA polymerase in isolated nuclei can be estimated from the rate of UMP incorporation upon addition of the exogeneous template, poly[d(A-T)] in the presence of actinomycin D. This activity is insensitive to actinomycin D. Engaged RNA polymerase on the other hand is associated with the rate of UMP incorporation which is due solely to the endogenous template. This activity is sensitive to actinomycin D. Since it has been established that there is negligible reinitiation of RNA synthesis in isolated macronuclei (Chapter 3), the macronuclear pools of free and engaged enzyme represent the in vivo levels which have become 'frozen' at the time of isolation of nuclei. This is actually only the case provided that the free RNA polymerase does not leak out from the macronuclei during isolation and the template engaged form is stable.

5.2 Results

(a) The effect of α-amanitin on RNA synthesis in isolated macronuclei

α-amanitin is a specific inhibitor of RNA polymerase II of mammalian tissues and lower eukaryotes which does not inhibit RNA

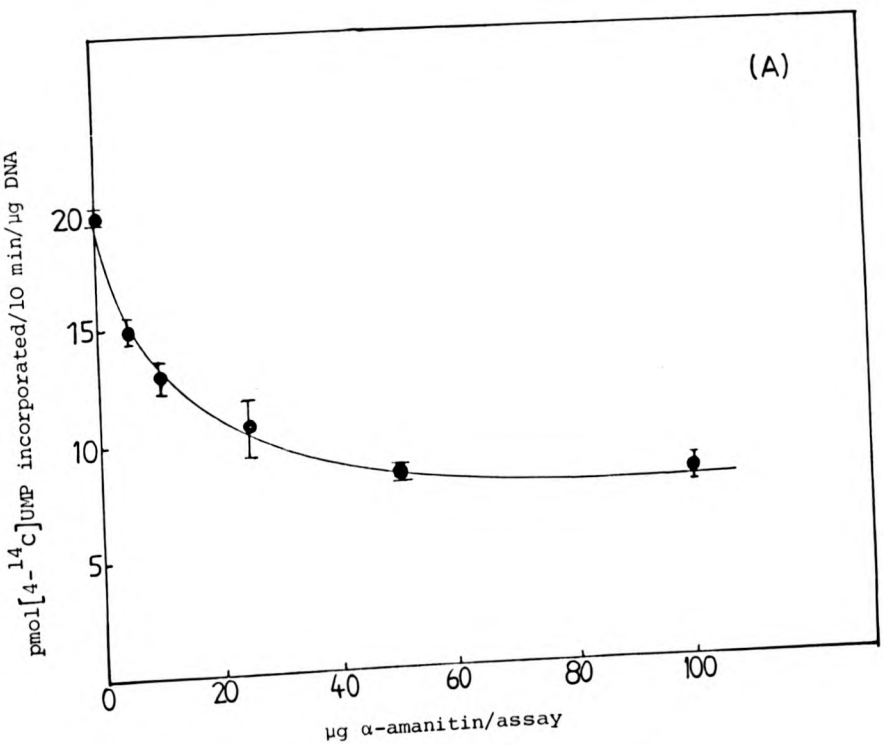
Figure 5.1 Inhibition of RNA synthesis in isolated macronuclei by α-amanitin

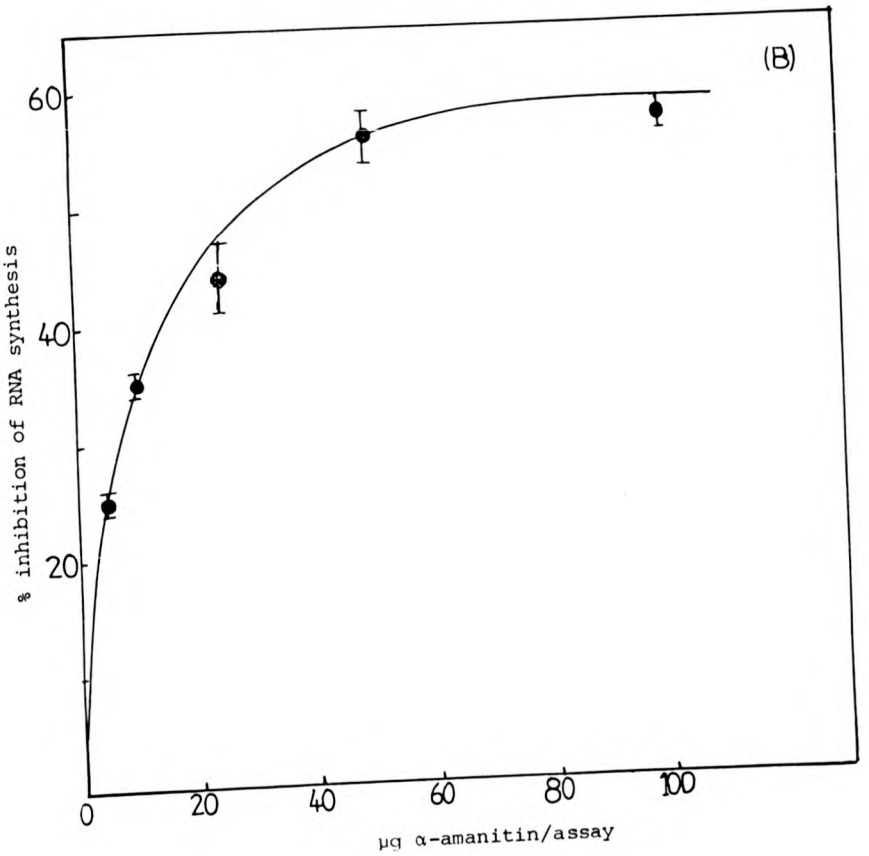
Nuclei were isolated from exponentially growing cells (3.5×10^5 cells/ml) and assayed for endogenous RNA polymerase activity as described in Section 2.7(a). α-amanitin was added to the incubation mixture at the concentrations indicated in the Figure. The reaction was started by the addition of macronuclei containing 35 μg nuclear DNA.

Curve (A) - represents RNA synthesis in the presence of α-amanitin

Curve (B) - represents the percentage inhibition of RNA synthesis by α-amanitin

[(n = 2), n = number of experiments performed]


(A)
pmol[4-14C]UMP incorporated/10 min/µg DNA
20
15
10
5
0
20
40
60
80
100
µg α-amanitin/assay


(B)
60
40
20
0
20
40
60
80
100
% inhibition of RNA synthesis
µg α-amanitin/assay

polymerase I. This inhibitor has been used in the present study to determine what percentage of the total RNA synthesis is due to the presence of RNA polymerase II in isolated macronuclei. The effect of a range of concentrations of α-amanitin on RNA synthesis is shown in Fig. 5.1A. Increasing concentrations progressively inhibit RNA synthesis until a plateau of inhibition is observed at concentrations greater than 50 µg/150 µl assay volume (333 µg/ml). We can deduce that at 50 µg/150 µl α-amanitin, almost all RNA polymerase II activity is inhibited, as has also been shown by Higashinakagawa (1975) with purified polymerase II. Fig. 5.1B, however, shows that about 56% of total RNA synthesis was inhibited at this concentration. Increase in α-amanitin concentration up to 100 µg had no further effect. Therefore we can conclude that this α-amanitin insensitive activity was due to RNA polymerase II.

(b) The effect of actinomycin D on RNA synthesis in isolated macronuclei

Fig. 5.2A shows that RNA synthesis in isolated macronuclei can be inhibited by actinomycin D. Low concentrations of the drug only partially inhibited RNA synthesis, but at a concentration of 167 µg/ml actinomycin D abolished the RNA synthetic capacity of macronuclei by more than 90% (Fig. 5.2B). This concentration was used in subsequent experiments when a complete block of endogenous activity was required.

(c) The use of poly[d(A-T)] as an exogenous template for RNA synthesis in isolated macronuclei

Fig. 5.3 shows that macronuclei incubated with the synthetic template poly [d(A-T)] have an increased capacity for RNA synthesis. The extent of stimulation is dependent on the amount of poly[d(A-T)] present in the assay mixture. The stimulation was not linear with increasing poly[d(A-T)] concentration, but appeared to be hyperbolic.

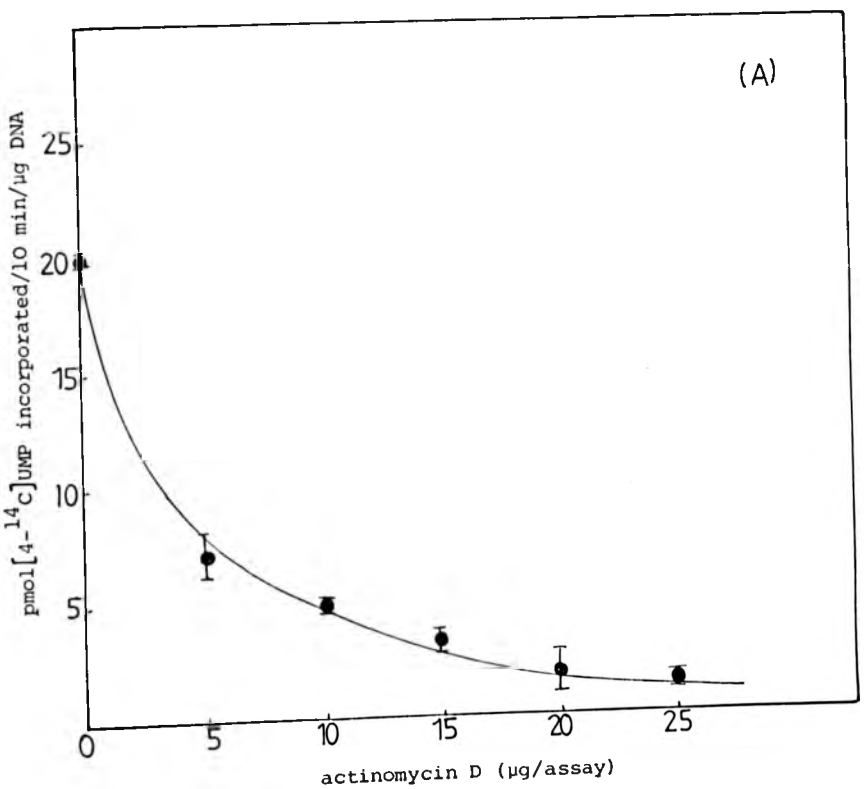
Figure 5.2 Inhibition of RNA synthesis in isolated macronuclei by actinomycin D

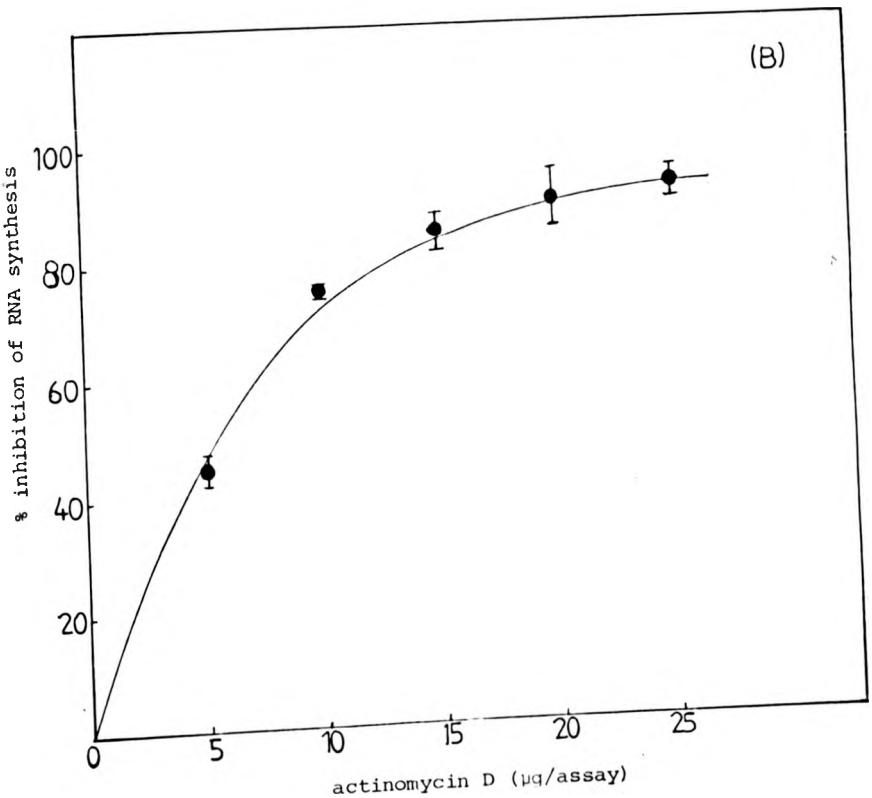
Nuclei were isolated from exponentially growing cells and assayed for endogenous RNA polymerase activity as described in Section 2.7(a). Actinomycin D was added to the incubation mixture at the concentrations indicated in the Figure. The reaction was started by the addition of macronuclei containing 40 µg DNA.

Curve (A) - represents RNA synthesis in the presence of actinomycin D

Curve (B) - represents the percentage inhibition of RNA synthesis by actinomycin D

[(n = 2), n = number of experiments performed]


(A)
pmol[4-14C]UMP incorporated/10 min/µg DNA
25
20
15
10
5
0
5
10
15
20
25
actinomycin D (µg/assay)


(B)
% inhibition of RNA synthesis
100
80
60
40
20
0
5
10
15
20
25
actinomycin D (µg/assay)

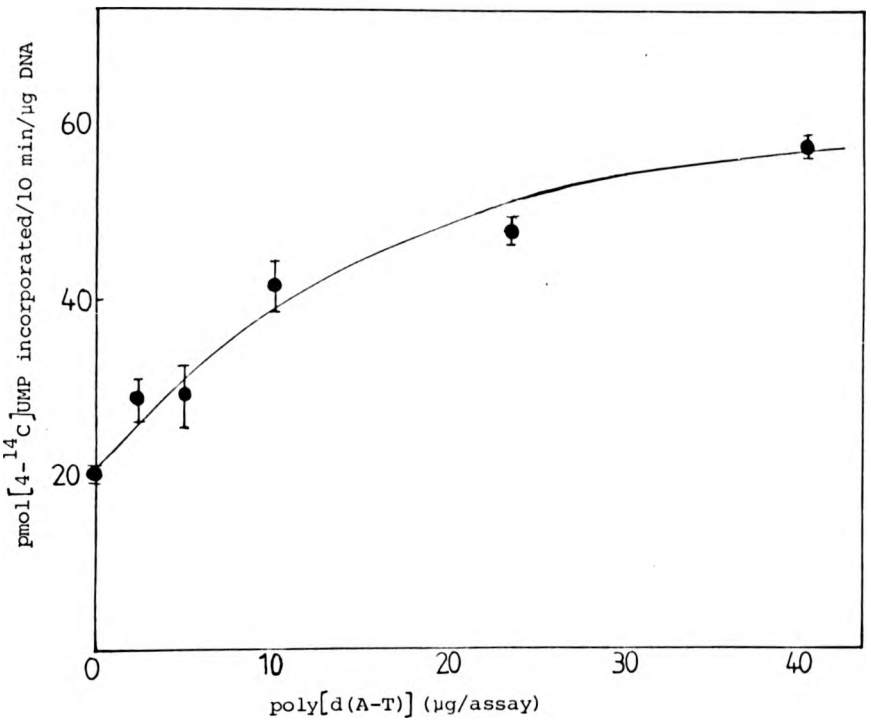


Figure 5.3 The effect of poly[d(A-T)] on RNA synthesis in isolated macronuclei

Nuclei were isolated from exponentially growing cells and RNA synthesis was measured according to the assay method described in Section 2.7(a). poly[d(A-T)] was added to the incubation mixture at the concentration indicated in the Figure. The reaction was started by the addition of macronuclei containing 40 µg DNA.

[(n = 2), n = number of experiments performed]

This was probably due to the saturation of the pool of free RNA polymerase molecules by an increasing concentration of template. For further studies, 30 µg/150 µl poly[d(A-T)] was used to give a measure of free RNA polymerase activity in isolated macronuclei.

(d) The effect of poly[d(A-T)] on RNA synthesis as a function of time

In the presence of exogenous template poly [d(A-T)] , the RNA synthesis increased with time,as can be seen from Fig. 5.4. The stimulation over control was found to be 1.8-fold.

(e) Evidence for free and engaged forms of RNA polymerase in macronuclei isolated from exponential cells

Actinomycin D was used to check whether the RNA polymerase which transcribes the added poly[d(A-T)] is enzyme which was dissociated from the transcription complex, or whether it represents excess free enzyme not active in endogenous transcription in isolated macronuclei. Assays were performed in the presence and absence of actinomycin D with and without added poly[d(A-T)] , (Table 5.1). Total RNA synthesis in the presence of this exogenous template was found to be the sum of the endogenous RNA synthesis plus the poly-[d(A-T)] directed synthesis measured at a high concentration of actinomycin D (167 µg/ml). This experiment provides important proof that the synthetic template was transcribed by a pool of free enzyme molecules. This basic experiment was done with macronuclei isolated by two different procedures - the Nonidet procedure and a procedure which did not involve the use of non-ionic detergents. Using these two types of macronuclei, the size of the pool of free RNA polymerase molecules measured by the poly[d(A-T)]/actinomycin D method was the same.

It can be concluded that the use of a detergent to prepare

Figure 5.4 RNA synthesis in isolated macronuclei in the presence of poly[d(A-T)] as a function of time

Macronuclei were isolated from exponential cells and RNA synthesis was measured as described in Section 2.7(a). 30 µg of poly[d(A-T)] was added to the reaction mixture. The reaction was started by the addition of macronuclei containing 35 µg DNA.

Curve (-■-■-) - represents RNA synthesis in the presence of poly[d(A-T)]
Curve (-●-●-) - represents RNA synthesis in the absence of of poly[d(A-T)]

[(n = 2), n = number of experiments performed]

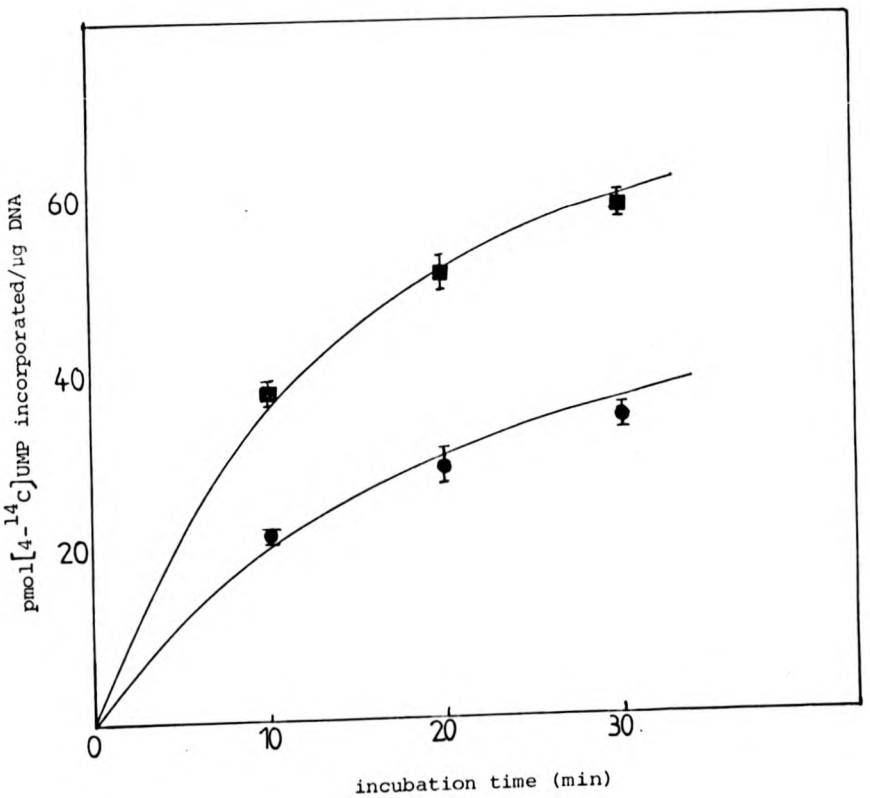

pmol[4-14C]UMP incorporated/µg DNA
60
40
20
0
10
20
30
incubation time (min)

Table 5.1 Evidence for free and engaged forms of RNA polymerase in macronuclei isolated from exponential cells

Incubation conditions	RNA polymerase activity (pmol of [4-^{14}C]UMP incorporated/10 min/ µg DNA)	
	Nonidet method	Non-detergent method
1. Macronuclei	20 ± 0.4	20 ± 1.2
2. + actinomycin D	2.0 ± 0.36	1.4 ± 0.1
3. + poly[d(A-T)]	42.4 ± 0.34	42 ± 0.6
4. + actinomycin D + poly[d(A-T)]	21.5 ± 2.8	22 ± 2.6

Macronuclei were isolated from exponential cells by the Nonidet method as described in Section 2.5(a) and by the Non-detergent method as described in Section 2.5(b). RNA synthesis was measured according to the assay methods described in Section 2.7 (a and b). Where indicated, 30 µg of poly[d(A-T)] and 25 µg of actinomycin D were added to the reaction mixture. The reaction was started by the addition of macronuclei containing 40 µg DNA.

[(n = 2), n = number of experiments performed]

macronuclei did not make them leaky with regard to the intranuclear pool of free RNA polymerase molecules.

(f) The effect of α-amanitin on free and engaged forms of RNA polymerase in isolated macronuclei

α-amanitin at the concentration of 50 µg/150 µl assay volume was used to distinguish between RNA polymerase I and II activities in isolated macronuclei. It can be seen from Table 5.2 that about 64% of the engaged RNA polymerase activity measured was due to RNA polymerase I. RNA synthesis in the presence of α-amanitin, poly-[d(A-T)] and actinomycin D also showed the presence of a pool of free RNA polymerase I molecules. It can be concluded from the results that both RNA polymerase I and II exist in free and engaged forms.

(g) Effect of heparin and rifamycin AF/103 on RNA synthesis using poly[d(A-T)] as exogenous template

Table 5.3 shows that in the presence of heparin or rifamycin AF/013 the stimulation of RNA synthesis by poly[d(A-T)] was abolished. RNA synthesis in the presence of exogenous template poly[d(A-T)] involved new initiation by free enzyme RNA polymerase molecules on the added template. The stimulation of endogenous RNA synthesis by heparin can be interpreted in terms of an increase in ribonucleotide elongation rates by pre-engaged RNA polymerase molecules in isolated macronuclei.

(h) Free and engaged forms of RNA polymerase in macronuclei isolated from starved cells

Exponentially growing cells were transferred from growth medium to buffered inorganic medium and starved for 24 hrs. Macronuclei were then isolated by the Nonidet method. Table 5.4 shows that macronuclei isolated from starved cells have a very low rate of endogenous RNA synthesis. The free RNA polymerase activity measured with poly[d(A-T)] in the presence of actinomycin D is also low compared to

Table 5.2 Effect of α-amanitin on free and engaged forms of RNA polymerase in isolated macronuclei

Incubation conditions	RNA polymerase activity (pmol of [4-^{14}C]UMP incorporated/10 min/μg DNA)
1. Macronuclei	20 ± 1.1
2. + α-amanitin	12.8 ± 0.7
3. + α-amanitin + actinomycin D	1.0 ± 0.01
4. + α-amanitin + poly[d(A-T)]	19.2 ± 1.2
5. + α-amanitin + actinomycin D + poly[d(A-T)]	7.1 ± 0.5

Macronuclei were isolated from exponential cells and RNA synthesis was measured according to the method described in Section 2.7(a). Where indicated, 50 μg of α-amanitin, 30 μg of poly[d(A-T)] and 25 μg of actinomycin D were added singly or in combination to the reaction mixture. Reaction was started by the addition of macronuclei containing 40 μg DNA.

[(n = 2), n = number of experiments performed]

Table 5.3 The effect of heparin and rifamycin AF/013 on RNA synthesis in isolated macronuclei

Incubation conditions	RNA polymerase activity (pmol of [4-^{14}C]UMP incorporated/10 min/µg DNA)
1. Macronuclei	20 ± 1.0
2. + poly[d(A-T)]	42.2 ± 0.63
3. + heparin	30.3 ± 0.34
4. + heparin + poly[d(A-T)]	30.4 ± 1.7
5. + rifamycin AF/013 + poly[d(A-T)]	19.2 ± 0.1

Macronuclei were isolated from exponential cells and RNA synthesis was measured according to the assay method described in Section 2.7(a). Where indicated, 30 µg of poly[d(A-T)], 150 µg of heparin and 9 µg of rifamycin AF/013 were added singly or in combination to the reaction mixture. The reaction was started by the addition of macronuclei containing 40 µg DNA.

[(n = 2), n = number of experiments performed]

Table 5.4 Free and engaged forms of RNA polymerase in macronuclei isolated from starved cells

Incubation conditions	RNA polymerase activity (pmol of [4-^{14}C]UMP incorporated/10 min/µg DNA
1. Macronuclei	3.5 ± 0.2
2. + actinomycin D	0.14 ± 0.02
3. + poly[d(A-T)]	9.0 ± 0.4
4. +actinomycin D + poly[d(A-T)]	7.2 ± 0.2

Tetrahymena cells were starved according to the method described in Section 2.6 and macronuclei were isolated by the Nonidet method (Section 2.5(a)). RNA synthesis was measured according to the methods described in Section 2.7 (a and b). Where indicated, 25 µg of actinomycin D or 30 µg of poly[d(A-T)]were added to the reaction mixture. The reaction was started by the addition of 38 µg DNA.

[(n = 2), n = number of experiments performed]

that present in macronuclei from rapidly dividing cells (Table 5.1). However, the ratio of free to engaged enzyme is about twice as great in starved cells compared to rapidly dividing cells.

5.3 Discussion

The results presented here have provided evidence for the existence of separate pools of free and engaged RNA polymerase in isolated macronuclei. The drugs actinomycin D, heparin and rifamycin AF/013 were used to show the transcription of poly [d(A-T)] by free RNA polymerase required de novo initiation, but was completely insensitive to actinomycin D. On the other hand, transcription by engaged enzyme was sensitive to actinomycin D but insensitive to initiation inhibitors. One problem in interpreting the results is associated with the question "Do the in vitro measurements on isolated nuclei represent the pool sizes of free and engaged enzyme in vivo?" Nuclei isolated by the detergent method using Nonidet have been examined under the electron microscope by Higashinakagawa and Mita (1973). They appear to have normal structure except that the outer nuclear membrane is absent. For this reason, nuclei were also prepared by a second method which did not make use of a non-ionic detergent. This method, originally devised by Bollinger, makes use of glycerol, a hypotonic buffer, to cause cells to swell, the cell membrane is broken by passage through a hypodermic syringe needle and the released nuclei are purified. Nuclei prepared by this method appeared to have almost identical pools of free and engaged enzyme to the nuclei prepared by the detergent method. Therefore we can deduce that the use of a non-ionic detergent does not cause any major leakage of free RNA polymerase. Furthermore, when nuclear washings were examined for free RNA polymerase activity using poly-[d(A-T)] as a template, no significant amount was found.

It might be predicted that the relative sizes of the two pools

of RNA polymerase in Tetrahymena macronuclei will vary with growth conditions. Measurement of the pool sizes under different conditions should allow one to determine if there is any role of these functional states of the enzyme in relation to regulation of RNA synthesis in the unicellular eukaryote. Such studies have already been done in other organisms:-

(i) Hentschel and Tata in 1977 have studied the free and template engaged RNA polymerase I and II activities in nuclei isolated from different developmental stages of the brine shrimp, Artemia salina. They demonstrated that transcriptionally active cells have a much lower proportion of free/engaged RNA polymerase than have the transcriptionally inactive dormant cells (encysted gastrulae).

(ii) Grummt et al. (1976) measured free and engaged RNA polymerase I activities in isolated nucleoli from ascites tumour cells which were grown in either complete or amino-acid (histidine) deficient medium. They observed that the amino-acid deficient cells had a higher proportion of free to engaged enzyme.

The pool sizes of free and engaged enzymes were therefore compared in exponential and starved Tetrahymena cells. Although the overall level of RNA synthesis was reduced to about 17%, the ratio of free to engaged enzyme was about twice as great in starved cells than in rapdily dividing cells. These results give some indication that as the pool size of engaged RNA polymerase goes down, the pool size of free RNA polymerase increases. Tetrahymena cells were starved by transfer of exponential cells to a simple buffered inorganic medium for 24 hrs. This method of starvation is probably fairly severe and produces a number of morphological as well as metabolic changes. However, the starved cells are motile and active. At the end of the starvation period, about 80-90% of the cells are stalled in the G1 phase of the cell cyle, and contain the normal

cellular amount of DNA for G1 cells (Mowat et al., 1974). The changes in the chromatin produced by starvation are interesting. The nucleus of transcriptionally inactive cells contains a high proportion of heterochromatin with a rather condensed structure, while that of active cells contains mostly euchromatin (Jeter et al., 1975). The dense heterochromatin is inactive in transcription, and this probably explains why starved cells have minimal synthetic activity.

CHAPTER 6

THE EFFECT OF CYTOPLASMIC EXTRACT ON RNA SYNTHESIS IN ISOLATED MACRONUCLEI

6.1 Introduction

There are problems in using isolated nuclei as an *in vitro* model system for studying transcription and its regulation. These are:- (i) in a suitable medium-containing substrate, transcription may last for 10 minutes, or with carefully prepared nuclei for one or two hours, but eventually comes to a stop. At this point further addition of substrate will not lead to further transcription; (ii) transcription in isolated nuclei is a process of elongation of existing RNA chains,and new initiations do not usually occur.

In a living cell, factors in the cytoplasm control the regulation of RNA synthesis in the nucleus. The classical experiments of Gurdon in 1967 on the effects of transplantation of differentiated *Xenopus* cell nuclei into egg cell cytoplasm established this hypothesis,and many other experiments since that time have confirmed it; for example, the cell fusion experiments of Harris (1967).

It therefore seemed logical to design experiments in which cytoplasm was added back to *Tetrahymena* macronuclei to see if transcription could be stimulated and eventually to determine if the cytoplasm contained any specific initiation factors. Such studies have been done in other systems:- (i) Bastian (1977) reported the stimulation of RNA synthesis in isolated nuclei from normal rat liver and from a rat hepatoma when they were incubated with cytoplasm obtained from homologous or regenerating rat liver; (ii) the stimulatory effect of added cytoplasm has also been demonstrated for isolated nuclei from *Xenopus* cultured cells (Crampton and

Woodland, 1979). They showed that the stimulatory factors were present in the cytoplasm of oocytes but absent from egg cell cytoplasm. RNA synthesis normally occurs at rapid rate in oocytes, but is minimal in egg cells. The overall stimulatory effect of cytoplasmic extracts was therefore changed during development and differentiation of Xenopus oocytes into mature egg cells. To date, no cytoplasmic factors capable of stimulating transcription have been reported for Tetrahymena. In this chapter, evidence is presented for the existence of such factors in the cytoplasm of exponential cells. A comparison is also made of the stimulatory effects of cytoplasm from exponential and stationary phase cells

6.2 Results

A cytoplasmic extract was prepared from exponential Tetrahymena cells by homogenising the cells and centrifuging the resultant homogenate at 100,000 g for 1 hr. This procedure removes all particulate matter including ribosomes from the cytosol. Table 6.1 shows that RNA synthesised by isolated macronuclei was stimulated in the presence of a cytoplasmic extract; however, the maximum stimulation obtainable over control was only 35%, even at very high concentrations of the extract (500 µg/150 µl assay volume). The concentration dependence curve is shown in Fig. 6.1. Dialysis of the cytoplasmic extract did not affect its capacity to stimulate transcription (Table 6.2), thus indicating that stimulation was not due to small molecules such as ions or nucleotides which may be present in the cytoplasm but which would pass through a dialysis sac. To check if there was any non-specific stimulation of transcription due to proteins in the cytoplasm, bovine serum albumin was added to the assay system. No significant stimulation was observed (Table 6.1). To determine how species specific was the stimulation of transcription, cytoplasm was prepared from two other eukaryote

Table 6.1 The effect of cytoplasmic extract and bovine serum albumin on RNA synthesis in isolated macronuclei

Incubation conditions	RNA polymerase activity (pmol of [4-^{14}C]UMP incorporated/10 min/ µg DNA)	% Stimulation over control
1. Macronuclei	19.4 ± 0.7	0
2. + cytoplasm	26.5 ± 1.0	36
3. + bovine serum albumin	20.4 ± 1.2	5

Macronuclei were isolated from exponential cells (3.5×10^5 cells/ml) as described in Section 2.5(a) and assayed for endogenous RNA polymerase activity as described in Section 2.7(a). Cytoplasm was prepared as described in Section 2.10(c) and used at a concentration of 500 µg protein/assay. Bovine serum albumin at the same concentration was used as indicated. The reaction was started at zero time by adding nuclei containing 40 µg DNA. DNA was determined as described in Section 2.11.

In the macronuclei used in control experiments, homogenisation buffer was used in place of cytoplasm.

[(n = 2), n = number of experiments performed]

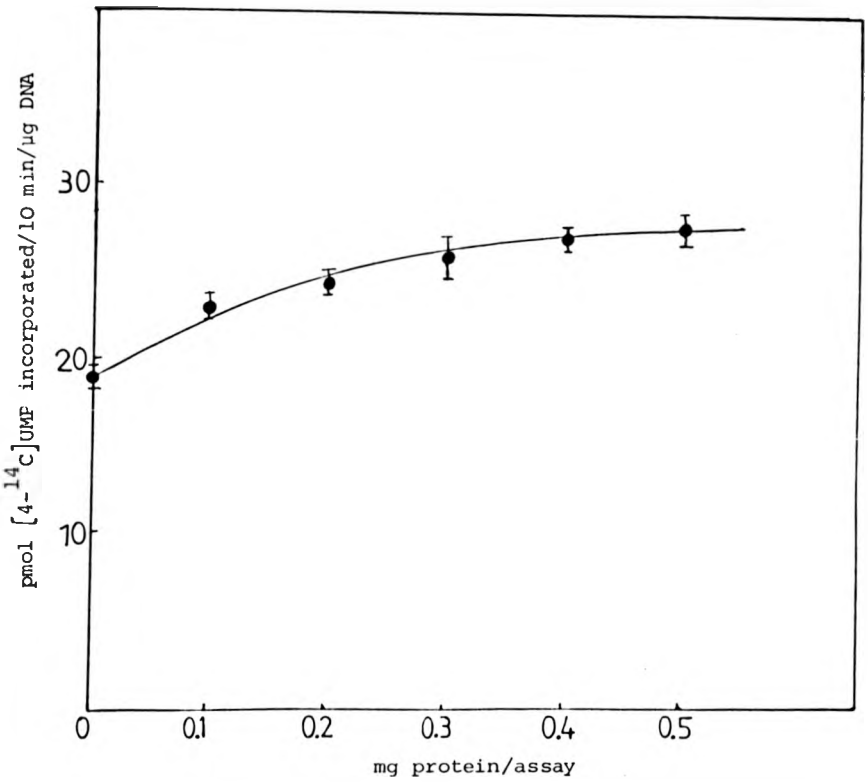


Figure 6.1 Concentration dependence of RNA synthesis on cytoplasmic protein in isolated macronuclei

Macronuclei were isolated from exponential cells as described in Section 2.5(a) and assay for endogenous RNA polymerase activity was carried out as described in Section 2.7(a). Cytoplasm was prepared as described in Section 2.10(a) and added to the incubation mixture at the concentrations indicated in the Figure. The protein concentration was determined according to the method described in Section 2.12. The reaction was started by the addition of macronuclei containing 40 µg DNA.

[(n = 2), n = number of experiments performed]

Table 6.2 The effect of dialysis on the stimulating effect of cytoplasmic extract in isolated macronuclei

Incubation conditions	RNA polymerase activity (pmol [4-^{14}C]UMP incorporated/10 min/ µg DNA)	% Stimulation over control
1. Macronuclei (control)	20.1 ± 0.7	0
2. + undialysed cytoplasm	27.2 ± 1.2	35
3. + dialysed cytoplasm	26.9 ± 1.4	33

Macronuclei were isolated from exponential cells as described in Section 2.5(a) and assayed for endogenous RNA polymerase activity as described in Section 2.7(a).

Cytoplasm was prepared as described in Section 2.10(c). Dialysis of the cytoplasm was carried out for 16 hours at 4° C against two changes of homogenising buffer (G) and dialysed cytoplasm was stored in aliquots at -70° C until used.

When required, undialysed and dialysed cytoplasm was added to the reaction mixture at a concentration of 500 µg protein/assay. The reaction was started at zero time by adding nuclei containing 40 µg DNA.

[(n = 2), n = number of experiments performed]

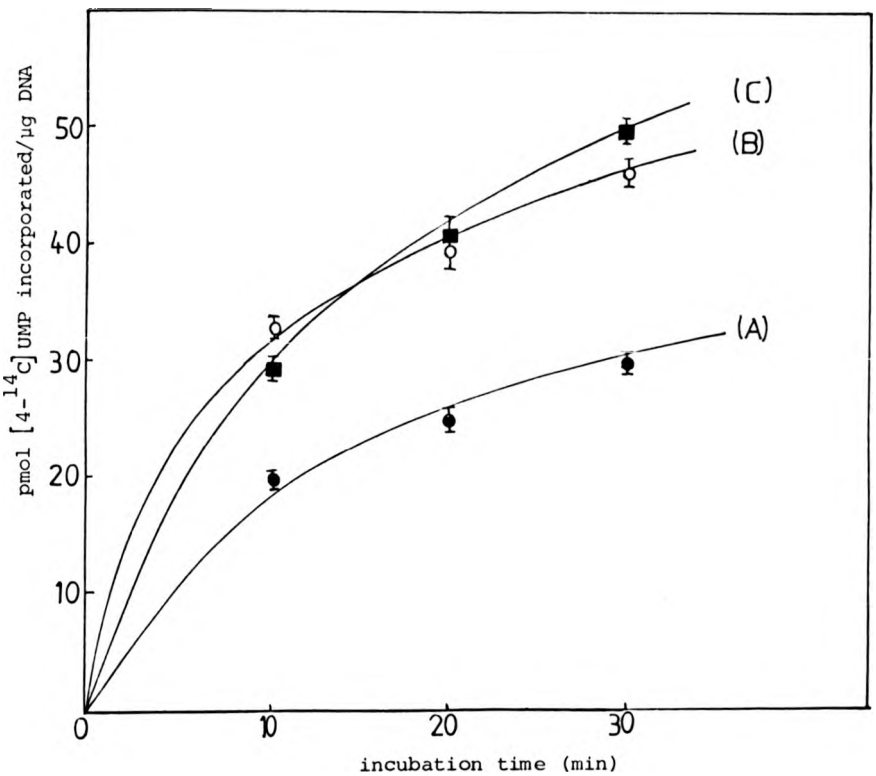


Figure 6.2 Effect of cytoplasm derived from different sources on RNA synthesis in isolated macronuclei

The experimental conditions used were the same as described in the legend for Table 6.1 except that:-

Cytoplasmic extract was prepared from chick embryo and Xenopus oocytes according to the method described in Section 2.10(a) and (b) respectively.

Curve (A) - represents RNA synthesis in the absence of cytoplasm

Curve (B) - represents RNA synthesis in the presence of cytoplasm from Xenopus oocytes

Curve (C) - represents RNA synthesis in the presence of cytoplasm from chick embryo

[(n = 2), n = number of experiments performed]

organisms, namely chick embryo and Xenopus oocytes. The cytoplasm from these organism also showed a stimulatory effect on RNA synthesis in isolated macronuclei of Tetrahymena. As can be seen from Fig. 6.2, equivalent amounts of cytoplasmic extract prepared from chick embryo produced a much higher stimulation than the extract from Tetrahymena. The extent of stimulation using 500 µg protein/150 µl assay was found to be 70%.Xenopus oocyte cytoplasmic extract gave a similar stimulation to that obtained with the cytoplasm from chick embryo. The stimulation of RNA synthesis by each of the cytoplasmic extracts was also studied as a function of time. The extract from chick embryo had a slightly more prolonged effect on RNA synthesis than that from oocytes.

To further investigate the specificity of the stimulation, a comparison was made of the effects of cytoplasmic extract from exponential and stationary phase cells. The extent of stimulation was studied as a function of time for both extracts and the results are shown in Fig. 6.3 In the presence of the cytoplasm from exponential cells, transcription was not only stimulated but appeared to proceed more linearly with time. This preliminary observation needs to be substantiated by measurements over a more extended time period than 30 minutes. Cytoplasm from stationary phase cells caused slight inhibition of RNA synthesis. One can therefore conclude that stimulatory factors are uniquely present in exponential cell cytoplasm and that these factors disappear as the cells approach stationary phase.

In the next group of experiments, I decided to use macronuclei from starved Tetrahymena cells in place of those from exponential cells for the following reason: exponential cells are very active in RNA synthesis while starved cells are not. Transcription in the exponential cells may already have been activated at or before

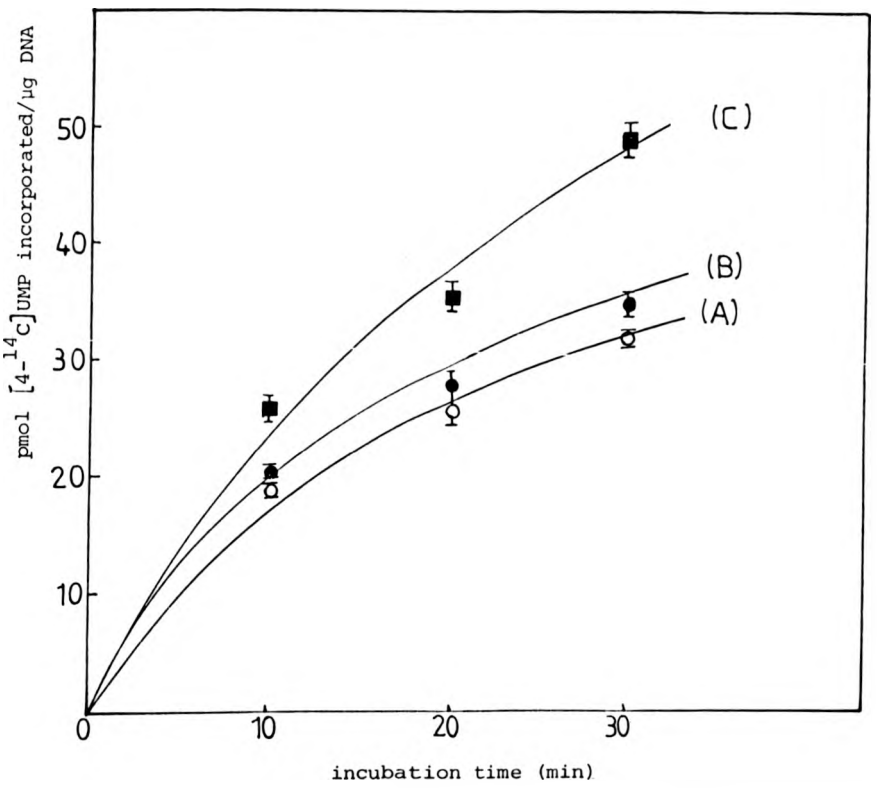


Figure 6.3 Effect of cytoplasmic extract obtained from exponential cells and stationary phase cells on RNA synthesis in isolated macronuclei

The exeprimental conditions used were the same as described in the legend for Table 6.1 except that:-

Cytoplasmic extract was prepared from exponential cells (3 x 10^5 cells/ml) and stationary phase cells (1 x 10^6 cells/ml) according to the method described in Section 2.10(c).

Curve (A) - represents RNA synthesis in the presence of cytoplasm from stationary phase cells

Curve (B) - represents RNA synthesis in the absence of cytoplasm

Curve (C) - represents RNA synthesis in the presence of cytoplasm from exponential cells

[(n = 2), n = number of experiments performed]

Table 6.3 The effect of cytoplasmic extract on the RNA synthesis in isolated macronuclei from starved cells

Incubation time	Incubation conditions	RNA polymerase activity (pmol [4-^{14}C]UMP incorporated/µg DNA)	% Stimulation over control
10 minutes	1. Macronuclei	3.5 ± 0.3	0
	2. + cytoplasm	6.65 ± 0.6	90
30 minutes	1. Macronuclei	4.5 ± 0.2	0
	2. + cytoplasm	9.4 ± 0.7	110

Macronuclei were isolated from starved cells as described in Section 2.6 and assayed for endogenous RNA polymerase activity as described in Section 2.7(a). Cytoplasm from Tetrahymena cells was prepared as described in Section 2.10(c) and when required added to the reaction mixture at the concentration of 500 µg protein/assay volume. The reaction was started at zero time by adding nuclei containing 40 µg DNA.

[(n = 2), n = number of experiments performed]

the time of the isolation of the macronuclei. When macronuclei from starved cells were incubated with cytoplasm from exponential cells, a major stimulation of endogenous transcription was observed. As shown in Table 6.3, the stimulation represented a 100% increase over the control. When studied as a function of time, added cytoplasm produced a more linear rate of RNA synthesis than seen in its absence. We can therefore conclude that cytoplasmic factors from exponential cells exert a major effect on the transcriptionally inactive macronuclei from starved cells.

6.3 Discussion

The results reported in the previous section show that factor(s) present in the cytoplasm of exponential cells stimulate the rate of RNA synthesis in isolated macronuclei. Various experiments have been designed to examine whether the stimulation is physiologically significant and associated with the regulation of RNA synthesis, or whether it has some trivial origin arising out of the use of an *in vitro* assay system. The stimulatory effect of *Tetrahymena* cytoplasmic extract was retained after extensive overnight dialysis of the cytoplasm against the homogenising buffer. So the possibility of the effect being due to small molecular weight molecules or an increase of the nucleotide pool can be ruled out. It was possible that proteins present in the cytoplasm might exert an effect by stabilizing the newly synthesised RNA *in vitro* and preventing its hydrolysis by ribonuclease. To test this possibility, bovine serum albumin was added to the assay mixture but,allowing for experimental error,it showed no stimulatory effect on RNA synthesis. An observation which points to the physiological significance of the effect is that the stimulatory factor(s) disappear from cytoplasm prepared from *Tetrahymena* cells, harvested in stationary phase. In fact, slight inhibition was observed when

cytoplasm from stationary phase cells was added to macronuclei from exponential cells. This could be explained by the presence of inhibitory factor(s) in the cytoplasm of these cells which reduces the RNA synthesised in isolated macronuclei. Recently Andersen and Neilsen (1979) have shown that Tetrahyemena cells approaching stationary phase secrete a low molecular weight factor which inhibits RNA synthesis in the stationary phase cells.

In general, stimulatory factors are found only in the cytoplasm of rapidly growing cells. This hypothesis is suppored by the observation that RNA synthesis is markedly increased when cytoplasm from chick embryo or Xenopus oocytes was incubated with isolated macronuclei from Tetrahymena. Such an effect of a heterologous cytosol in transcription was also observed by Bastian (1977), using nuclei isolated from rat liver. These results suggest that at least some of the stimulatory factor(s) are neither species nor order specific. Similar conclusions have been drawn from in vivo cell fusion experiments, using cells from different species.

In some experiments, macronuclei isolated from starved cells have been used, because of the possibility that macronuclei isolated from exponential Tetrahymena cells are already activated by endogenous factors. Macronuclei from starved cells have a low basal level of RNA synthetic activity, and when these macronuclei were incubated with cytoplasm derived from exponential cells, a major stimulation was observed in the rate of RNA synthesis. The stimulation was in the order of 100%. These results are in good agreement with the study made by MacLean and Hilder in 1977. They reported the stimulation effect of a cytoplasmic extract derived from rat liver on isolated nuclei from Xenopus erythrocytes. They showed that these nuclei were essentially inactive in RNA synthesis

but could be reactivated in the presence of cytoplasmic extract. The stimulatory factor in this case was found to be a low molecular weight protein.

The *in vitro* studies reported in this chapter confirm that cytoplasm does modulate RNA synthesis in isolated macronuclei. However, before a mechanism can be postulated, purification of these factors is the next essential stage before their role can be established. The problems associated with this type of work will be discussed in the final chapter.

CHAPTER 7

GENERAL DISCUSSION

The aims of this project were set out at the end of the introduction. The purpose of this chapter is to review to what extent these aims have been fulfilled, draw together conclusions and also to predict the direction of future research on the control of transcription in Tetrahymena.

In the work reported here, isolated macronuclei have been used exclusively as a model in vitro system for the study of RNA synthesis. The initial aims of the project were to test how good a model system they represent when compared to in vivo transcription. The tests were formulated in terms of four basic questions:-

(1) Does initiation occur in isolated macronuclei?

(2) What is the size and nature of the in vitro RNA products?

(3) How much RNA polymerase is template engaged and how much is free under various physiological conditions? What controls the ratio of free to engaged enzyme in vivo and in vitro?

(4) Are control factors for transcription present in cytoplasm? How do such factors affect transcription by isolated nuclei?

(1) Initiation in isolated macronuclei.- Using the initiation inhibitors rifamycin AF/013 and heparin we were forced to come to the conclusion that isolated macronuclei are not capable of reinitiating RNA synthesis. They do appear to be able to elongate RNA chains that were initiated in vivo and presumably they can terminate newly synthesised RNA chains which can be transported out of the nucleus. Very recently it was proposed that Tetrahymena has special termination factor(s) present in the macronuclei which are required for the correct termination of preribosomal RNA. These termination factor(s) were initially

extracted from nucleoli at high ionic strength, using 100 mM $(NH_4)_2SO_4$. In the absence of termination factor, RNA polymerase I tended to overshoot in the transcription of ribosomal DNA and produced a transcript that was greater in size than seen under *in vivo* conditions (Leer *et al.*, in press). It is probable that the isolated macronuclei used in this project were capable of correct termination. In the first place they were not normally treated with high salt concentrations and secondly they appeared to produce two products which have the same size as *in vivo* 17S and 25S rRNA. The release of newly synthesised RNA from macronuclei was investigated in a single preliminary experiment. It was found that after 10 minutes incubation with substrates, 15% of the RNA products were found outside the nucleus, while 85% was still retained inside. This result points to a rather slow release of RNA from the nucleus or may be due to an incomplete transport system *in vitro* (Ishikawa *et al.*, 1978).

When we consider the reasons why isolated macronuclei are not capable of reinitiation, two possible explanations spring to mind:-

(i) In the isolation of macronuclei, initiation factors could be lost which can easily diffuse in and out of the nucleus from the cytoplasm.

(ii) In the isolation of macronuclei, structural damage could occur to one or other of the components involved in transcription, such as the chromatin, which then prevents initiations from taking place.

In the situation where it is wished to detect a small amount of initiation in the presence of a high background of RNA elongation, it is best to choose a technique which only monitors initiation. The ideal technique for doing this has been recently developed. It will be discussed here in some detail because it could be used

to definitively answer the question "does cytoplasm stimulate the initiation of new RNA chains in isolated macronuclei?" The technique, described by Smith et al. in 1978, makes use of either adenosine or guanosine 5'[γ-S] triphosphates as affinity probes for studying RNA chain initiation. Transcripts, newly initiated with either nucleotide analogue are isolated by affinity chromatography on a mercury-agarose affinity column. The binding is specific and only dependent upon the inclusion of the sulphur nucleotide analogues in the in vitro synthetic reaction. The important feature of the technique is that those chains which are elongated but not initiated in an in vitro experiment are not retained on the column. The newly initiated chains can be eluted from the column by the addition of dithiothreitol to the chromatography buffer, and the chains can be characterised as having 5'[γ-S] triphosphate at their 5' terminal end. This is done by hydrolysing them with alkali and showing that a single nucleoside 5'[γ-S] triphosphate 2',3'-monophosphate can be recovered in high yield. Using this technique, Smith et al. proved conclusively that 5S RNA can be accurately initiated in vitro experiments with isolated mouse myeloma nuclei.

2. The size and other properties of in vitro synthesised RNA

It has been established that, given the right incubation conditions, macronuclei can synthesise discrete species of RNA. So far these RNA species have been characterised only in terms of their size. This has been estimated by the use of markers and a simple formula relating electrophoretic mobility to molecular weight and 'S value' (Spirin, 1963). The species of RNA which have been observed are 17S, 25S, 4-5S and 8-15S, together with one or two additional minor species. By comparison with the sizes of RNA which can be observed in in vivo experiments, these species

are tentatively identified as:-

17S
25S — fully processed ribosomal RNA

33S, 20S and 28S — possible precursors of ribosomal RNA

4-5S — transfer RNA and 5S ribosomal RNA

8-15S — messenger RNA

The last species identified as putative mRNA is seen as a massive discrete peak in gel electrophoresis only when heparin is included in the incubation mixture.

It is obvious that the measurement of size of in vitro synthesised RNA is only a simple initial way of characterizing these products,and for rigorous identification of each species one needs to measure other properties. The additional tests that should be used in further studies are

(1) identification of each species of RNA by hybridisation with its complementary DNA (provided that this can be easily isolated). It is in fact not difficult to isolate the complementary DNA for ribosomal RNA, since in Tetrahymena this exists as a discrete, gene amplified, extra-chromosomal species which can be purified from nucleoli;

(2) identification of processed mRNA by its special features at the 3' and 5' terminal ends. Isolated nuclei in general contain enzymes which will methylate specific residues at the 5' end and add a poly A chain to the 3' end of a newly synthesised mRNA molecule. mRNA molecules with poly A tails can be specifically adsorbed on a column of oligo dT attached to a solid support. They can thus be very easily identified as a class of molecules, although the identification of specific species of mRNA is a more difficult task;

(3) identification of in vitro synthesised mRNA or rRNA by its ability to perform a function. This is the most important test to determine if an in vitro product is essentially the same as that

produced in a living cell. If individual species of mRNA can be obtained from an *in vitro* experiment, they can then be tested for their ability to code for a functional protein in either (a) the rabbit reticulocyte or (b) the wheat germ translational system. At this time, there is only one clear-cut example of the *in vitro* synthesis of characterised mRNA in the literature. Manley *et al.* (1979) have analysed the RNA produced *in vitro* by incubating nuclei from HeLa cells infected with adenovirus. They showed that adenovirus-specific mRNA is produced at a linear rate for up to 2.5 hours. They were able to prove by sequence analysis of the 5' termini of their *in vitro* transcripts that their system initiates RNA chains *de novo* at the correct promoter, and that the 5' terminus is capped (methylated at the correct position).

3. Free and engaged enzyme in isolated nuclei

It has been shown that Tetrahymena contains separate pools of free and template engaged RNA polymerase in its macronuclei. Analysis with α-amanitin has indicated that both RNA polymerase I and II show this behaviour.

Initial experiments have been performed to show that the physiological state of the cell (growth conditions) affects the pool size of the different forms of the enzyme. Thus starved cells had a greater ratio of free to engaged than exponential cells. In general, it might be expected that cells which were growing rapidly would have relatively more of their RNA polymerase in the template engaged form. An important question then arises as to what controls the ratio of free to engaged enzyme. Some observations, recently reported by Yukioka *et al.* (1979) appear to shed some light on this problem. Engaged enzyme is complexed to chromatin so tightly that very drastic techniques have usually been used to solubilize it, *i.e.* sonication in high ionic strength media. To avoid this problem, Yukioka *et al.* devised a gentle treatment to disrupt rat liver nuclei

and release engaged RNA polymerase. The nuclear suspension was digested with 250 units/ml of micrococcal nuclease for 2.5 minutes at 37° C and the digestion was terminated by cooling on ice and adding EGTA to a final concentration of 0.5 mM. Using this technique, they were able to show that the micrococcal nuclease digest of rat liver chromatin contained two species of RNA polymerase II which could be easily distinguished from each other. These could be separated by sucrose density centrifugation or DEAE-sephadex chromatography. One species of RNA polymerase II contained a factor which enhanced the transcription of a chromatin template. If this species was treated with 0.5 M NaCl prior to sucrose density centrifugation, it appeared to be entirely converted to a form lacking the factor and having a different activity with DNA templates. So far Yukioka et al. have not reported any detailed characterisation of their factor and this appears to be the next stage of further studies. Future work on the control of the pool size of free and engaged enzyme must obviously take into account these interesting observations.

4. Transcription and the interaction between nuclei and cytoplasm

It was found that transcription in isolated macronuclei from exponential cells could be stimulated by the addition of cytoplasm from rapidly dividing cells. The stimulation, however, was not very great and amounted to only 35% above control. Cytoplasm from stationary phase cells on the other hand slightly inhibited transcription. The same general effects were seen when nuclei from starved cells were used, except that a greater percentage stimulation was seen on the addition of cytoplasm from exponential cells (100%). These stimulatory effects are obviously not entirely species specific, since cytoplasm from Xenopus oocytes and chick embryo can also stimulate transcription in Tetrahymena macronuclei

(approximately 80%). These latter observations on an *in vitro* system are consistent with the results of Harris (1970) on *in vivo* RNA synthesis in heterokaryons formed by fusing together two different cell types. The heterokaryons were formed by fusing together unlike cell types, using 'Sendai" virus to form hybrid cells in which two different types of nuclei are present in one common cytoplasm. Harris observed that if either of the parent cells normally synthesised RNA, then RNA synthesis will take place in both types of nuclei in the heterokaryon. Thus hen erythrocyte cells do not synthesize RNA any more, but in a heterokaryon with HeLa cells, RNA synthesis is switched back on again. It was concluded that the hybrid cytoplasm transmits signals to the genes of the erythrocyte, causing it to restart RNA synthesis. The results reported here on the quite different effects of cytoplasm of exponential and stationary phase cells can also be compared with the *in vitro* studies of Crampton and Woodland (1979 a and b) on *Xenopus laevis*. They found that extracts of full-grown oocytes stimulated the rate of RNA synthesis 2.5 fold and caused it to continue linearly for at least 6 hours when nuclei were pre-incubated with cytoplasm and nucleotides. The stimulatory agents in the cytoplasmic extract disappeared in the egg, remained absent through cleavage but reappeared in the late blastula stage. These results correspond to changes in the RNA synthesis that occur during the early development of *Xenopus*.

In the work on *Tetrahymena*, the obvious next stage is to fractionate the cytoplasm of exponential cells (and stationary phase cells) to find out (i) how many different types of stimulatory factor are present, (ii) to determine at which stage of RNA synthesis they act, *i.e.* initiation, elongation or termination, or (iii) to determine how specific is each factor

isolated. In the case of the Xenopus system, a start has been made on answering these questions. Oocyte extracts were fractionated on G-100 Sephadex and DEAD-cellulose chromatography. Four different activities were found which stimulate RNA synthesis in isolate of Xenopus nuclei. Three factors affect all polymerase activities, producing stimulations of five to eight fold. The fourth specifically stimulates rRNA synthesis by over 20 fold. This rRNA specific factor clearly increased the rate of initiation, and it and one of the non-specific factors are greatly reduced in extracts of eggs. These two stimulatory factors return to normal levels at the late blastula stage of development, and remain abundant through later stages.

Although much work still remains to be done on the Tetrahymena system, I hope that I have shown in this thesis that it is an interesting system, potentially capable of yielding much new information on the control of transcription.

APPENDIX

Peacock and Dingman (1968) have used the acrylamide-agarose composite gels to separate RNA species of different molecular weights. They have shown that the mobility and logarithm of molecular weight are inversely related, and this relationship is approximately linear.

To characterise the RNA species synthesised in Tetrahymena macronuclei, five standard RNA species were used. These species are total E. coli RNAs (23S, 16S and 4S) and Xenopus ribosomal RNAs (28S and 18S). Using Spirin's empirical formula ($M = 1550XS^{2.1}$), the molecular weights of RNA species can be converted to S values (Spirin, 1963).

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